Organisation for Economic Co-operation and Development

ENV/CBC/MONO(2021)14

Unclassified

English - Or. English

8 July 2021

ENVIRONMENT DIRECTORATE
CHEMICALS AND BIOTECHNOLOGY COMMITTEE

VALIDATION STUDY REPORT AND INDEPENDENT PEER-REVIEW REPORT FOR INCLUSION OF KERASKIN SKIN IRRITATION TEST TO THE TEST GUIDELINE 439 ON IN VITRO SKIN IRRITATION: RECONSTRUCTED HUMAN EPIDERMIS TEST METHOD

Series on Testing and Assessment,
No. 339

.

JT03479329

SERIES ON TESTING AND ASSESSMENT

NO. 339

VALIDATION STUDY REPORT AND INDEPENDENT PEER-REVIEW REPORT FOR INCLUSION OF KERASKIN SKIN IRRITATION TEST TO THE TEST GUIDELINE 439 ON IN VITRO SKIN IRRITATION: RECONSTRUCTED HUMAN EPIDERMIS TEST METHOD

IOMC

INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

A cooperative agreement among FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD

Environment Directorate
ORGANISATION FOR ECONOMIC COOPERATION AND DEVELOPMENT
Paris 2021

About the OECD

The Organisation for Economic Co-operation and Development (OECD) is an intergovernmental organisation in which representatives of 36 industrialised countries in North and South America, Europe and the Asia and Pacific region, as well as the European Commission, meet to co-ordinate and harmonise policies, discuss issues of mutual concern, and work together to respond to international problems. Most of the OECD's work is carried out by more than 200 specialised committees and working groups composed of member country delegates. Observers from several countries with special status at the OECD, and from interested international organisations, attend many of the OECD's workshops and other meetings. Committees and working groups are served by the OECD Secretariat, located in Paris, France, which is organised into directorates and divisions.

The Environment, Health and Safety Division publishes free-of-charge documents in eleven different series: **Testing and Assessment**; **Good Laboratory Practice and Compliance Monitoring**; **Pesticides**; **Biocides**; **Risk Management**; **Harmonisation of Regulatory Oversight in Biotechnology**; **Safety of Novel Foods and Feeds; Chemical Accidents; Pollutant Release and Transfer Registers; Emission Scenario Documents;** and **Safety of Manufactured Nanomaterials.** More information about the Environment, Health and Safety Programme and EHS publications is available on the OECD's World Wide Web site (www.oecd.org/chemicalsafety/).

This publication was developed in the IOMC context. The contents do not necessarily reflect the views or stated policies of individual IOMC Participating Organizations.

The Inter-Organisation Programme for the Sound Management of Chemicals (IOMC) was established in 1995 following recommendations made by the 1992 UN Conference on Environment and Development to strengthen co-operation and increase international co-ordination in the field of chemical safety. The Participating Organisations are FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD. The purpose of the IOMC is to promote co-ordination of the policies and activities pursued by the Participating Organisations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

This publication is available electronically, at no charge.

Also published in the Testing and Assessment link

For this and many other Environment, Health and Safety publications, consult the OECD's World Wide Web site (www.oecd.org/chemicalsafety/)

or contact:

OECD Environment Directorate,
Environment, Health and Safety Division
2 rue André-Pascal
75775 Paris Cedex 16
France

Fax: (33-1) 44 30 61 80

E-mail: ehscont@oecd.org

Foreword

This document contains the validation study report (Part 1) and the independent peer-review report for KeraSkin™ Skin Irritation Test (SIT) (Part 2), a me-too assay for the **OECD Test Guideline (TG) 439.**

TG 439 was updated in 2021 with the inclusion of a new test method, KeraSkin™ SIT. The update of the TG was led by Korea, with the support of the expert group on Skin and Eye Irritation. The validation study was intented to assess reliability (the within- and between- laboratory reproducibility) and relevance (the predictive capacity) of KeraSkin™ SIT in accordance with the performance standard (PS) for OECD TG 439 as described in OECD Guidance Document 220. The validation study was conducted by 5 laboratories in Korea from April 2018 – December 2019. The independent peer-review report provides technical questions that were raised by the review panel from the expert group and the responses for them from the test developers. The process to prepare the independent peer-review report took place from March 2020 – July 2020.

The validation study report was circulated for a review from the Working Group of the National Coordinators of the Test Guidelines Programme (WNT), along with the updated TG 439 in December 2020. All comments received were addressed by Korea and reflected on the documents accordingly. The WNT approved the validation study report and the independent peer-review report at the 33rd WNT meeting in April 2021. This document is published under the responsibility of the Chemicals and Biotechnology Committee.

PART 1

KeraSkin™ SIT Validation Report

Validation study for *in vitro* skin irritation test with 3D-reconstructed human epidermis model, KeraSkin™

8th February 2021

Contents

List of Appendices

List of Authors

Korean Center for the Validation of Alternative Methods (KoCVAM)

Dr. Yoon, Hae Seong
Dr. Kim, Tae Sung
Dr. Kim, Hak
Ms. Lee, Ga Young

Validation Management Team

Dr. Chun, Young Jin (Chung-Ang Univ.)
Dr. Son, Youngsook (Kyunghee Univ.)
Dr. Kim, Bae-Hwan (Keimyung Univ.)
Dr. An, Susun (AmorePacific)
Dr. Yun, Jun Won (Catholic Univ.)
Dr. Yoon, Hae Seong (KoCVAM)

Lead laboratories

Dr. Lim, Kyung-Min
College of Pharmacy, Ewha Womans University, 52 Ewhayeodae-gil, Seodaemun-gu, Seoul, Republic of Korea

Dr. Lee, Su Hyun
Biosolution Co., Ltd
Room 519, Seoul Technopark,
232 Gongneung-ro, Nowon-gu, Seoul,
Republic of Korea

Data Analysis Group

Dr. Bae, Seung Jin
College of Pharmacy, Ewha Womans University, Republic of Korea

Participating Laboratories

Dr. Lee, Su Hyun
Biosolution Co., Ltd
Room 519, Seoul Technopark,
232 Gongneung-ro, Nowon-gu, Seoul,
Republic of Korea

Dr. Kim, Jin Sik & Dr. Kim, Ki Hun
Korea Conformity Laboratories,
8 Gaetbeol-ro 145 Beon-gil, Yeonsu-gu,
Incheon, Republic of Korea

Ms. Chang, Yu Jin
COSMAX Korea,
Building E, Pangyo Innovalley, 255
Pangyo-ro, Bundang-gu, Seongnam-si,
Gyeonggi-do, Republic of Korea

Dr. Jung, Mi Sook
Biotoxtech Co., Ltd.,
Safety Evaluation Team,
686-2 Yangcheong-ri, Ochang-eup,
Cheongwon-gu, Cheongju-si, Chungbuk,
Republic of Korea

Acknowledgements

Ms. Lim, Song E & Ms. Han, Joo Hee
College of Pharmacy,
Ewha Womans University, Republic of Korea

List of Acronyms and Abbreviations

BLR	Between-Laboratory Reproducibility
CAS	Chemical Abstracts Service
CST	Chemical Selection Team
DPBS	Dulbecco's Phosphate Buffered Saline
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals
ET_{50}	Effective Time 50
EURL-ECVAM	European Union Reference Laboratory–European Centre for the Validation of Alternative Methods
GHS	Globally Harmonized System of Classification and Labelling of Chemicals
GLP	Good Laboratory Practice
IC_{50}	Inhibitory Concentration 50
KoCVAM	Korean Center for the Validation of Alternative Methods
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
OD	Optical Density
OECD	Organization for Economic Cooperation and Development
PS	Performance Standards
QA	Quality Assurance
QC	Quality Control
RhE	Reconstructed Human Epidermis
ROC	Receiver Operating Characteristic
SD	Standard Deviation
SIT	Skin Irritation Test
SOPs	Standard Operating Procedures
TG	Test Guideline

VMT	Validation Management Team
VRM	Validated Reference Method
WLR	Within-Laboratory Reproducibility
WNT	Working Group of the National Coordinators of the Test Guidelines Programme

Definitions

Accuracy: The closeness of agreement between test method results and accepted reference values. It is a measure of test method performance and one aspect of relevance.

ET_{50}: The time of exposure to 1.0 % Triton X-100 estimated to decrease the viability by 50%

IC_{50}: The concentration (mg/mL) of SDS estimated to reduce the viability by 50%

Lead laboratory: The laboratory responsible for training other participating facilities involved in standardization, optimization, and validation of a test method.

Project plan: A validation study plan designed to help participants understand the validation study by providing essential information and describing responsibilities and duties of each participating party.

Protocol: A test plan that clearly details each step of a validation method and provides criteria and a process to prepare reagents, supplies, and tools to generate test data.

Reference chemicals: Chemicals that have already been validated in other test systems and species and can be selected for use in the validation process.

Relevance: Description of the relationship of the test to effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test currently measures or predicts the biological effect of interest. Relevance incorporates consideration of accuracy of a test method.

Reliability: The extent to which a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol.

Reproducibility: The agreement among results obtained from testing the same substance using the same test protocol.

Run: A run consists of one or more test chemicals tested concurrently with a negative control and a positive control.

Sensitivity: The proportion of all positive/ active substances that are correctly classified by the test.

Specificity: The proportion of all negative/ inactive substances that are correctly classified by the test.

Standard Operating Procedures (SOPs): A document that describes specific tests and the process of laboratory operation.

Transferability: The extent to which an independent testing facility can accurately and reliably perform a test procedure.

Validation: A process to demonstrate the reliability and relevance of an alternative test method.

List of Tables

List of Figures

I. Goal Statement

The KeraSkin™ Skin Irritation Test (SIT) validation study was intended to assess reliability (the within- and between-laboratory reproducibility) and relevance (the predictive capacity) of KeraSkin™ SIT in accordance with the performance standards (PS) for OECD TG439 as described in OECD GD 220 (OECD, 2015a).

II. Objective

The OECD WNT (Working Group of the National Coordinators of the Test Guidelines Programme) adopted a new *in vitro* test, OECD TG 439, to assess skin irritation using a reconstructed human epidermis (RhE) tissue model in 2010 (OECD, 2015c). At the time of original adoption, EpiDerm™ SIT and EpiSkin™ SIT were only two VRMs approved for OECD TG 439. Then, PS based skin irritation tests, SkinEthic™ RhE SIT and Labcyte EPI-MODEL24 SIT were developed, validated and approved for OECD TG 439 in 2013. In 2019, OECD TG 439 was updated with two additional PS based skin irritation test methods, epiCS® and Skin+® (OECD, 2019). The KeraSkin™ SIT is a new *in vitro* test method for evaluating skin irritation using a new RhE manufactured with primary human keratinocytes (Jung et al., 2014). The purpose of this validation study was to evaluate the reproducibility of within-laboratory and between-laboratory, and the predictive capacity (sensitivity, specificity, and accuracy) for ensuring that the KeraSkin™ SIT meets the standards for a PS based test stated in OECD GD 220 (OECD, 2015a). This validation study was carried out in accordance with the OECD GD 220 performance standards published in 2015 as well as the modular approach as described in OECD Guidance Document 34 (OECD, 2005). And to further evaluate the predictive capacity of KeraSkin™ SIT, the data for 66 test chemicals in total that were compatible with the current protocol, were generated

and analyzed.

III. Background

Since the EU ban on animal testing for cosmetics in 2013, more than 37 countries around the world including the Republic of Korea have legally prohibited animal experiments for the development of cosmetics (Akbarsha & Mascarenhas, 2019). Accordingly, *in vitro* skin irritation tests have been developed to replace OECD TG 404 acute irritation/corrosion test (OECD, 2015b), which has been used to evaluate the skin irritation of chemicals, and the following test guideline was approved as OECD TG;

TG 439: *In Vitro* Skin Irritation : Reconstructed Human Epidermis Test Method (OECD, 2015c)

RhE SIT is capable of distinguishing between skin irritants (UN GHS Category 2) and non-irritants (No Category), and it can also be used in combination with other replacement methods to enable further classification of the hazard on skin in the framework of an Integrated Approach on Testing and Assessment (IATA) (OECD, 2017).

The KeraSkin™ model is a newly developed RhE model, which is reconstructed using primary cultured human keratinocytes from residual foreskin after circumcision. Its morphological microstructure and biomarker expressions are similar to those of human skin epidermis (Jung et al., 2014). Studies have been conducted to develop new *in vitro* assays for evaluating skin irritation using KeraSkin™, and the final protocol for KeraSkin™ SIT was completed with several protocol refinements through pre-validation and the current validation study. The applicability of KeraSkin™ SIT in assessing skin hazard has been demonstrated in several papers published in international journals (Choi et al., 2014; Hwang, Park, Choi, Nam, & Lim, 2018; Jung et al., 2014; Kim et al., 2017).

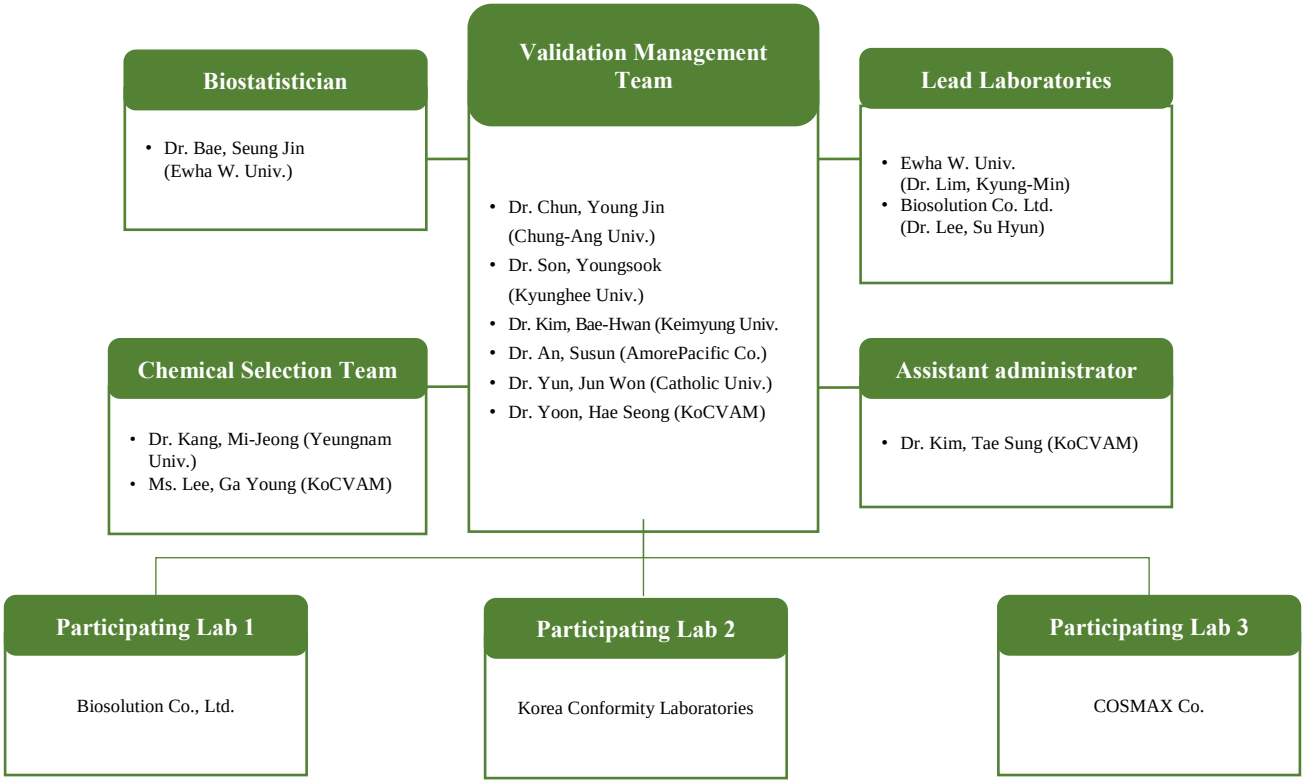
This validation study was conducted under the steering of a validation management team.

IV. Reconstructed Human Epidermis Model, KeraSkin™

The KeraSkin™, a multilayered (≥ 4 layers) epidermis model composed of stratum corneum, stratum granulosum, stratum spinosum and stratum basale, was developed by Biosolution Co., in Korea. The KeraSkin™ model was prepared from human keratinocytes isolated from residual foreskin tissues remaining after circumcision under the approval of Chung-Ang University Hospital IRB (#10-049-07-13). Donors were male and their ages ranged from 10 to 17. Each batch was from a single donor and karyotyping was conducted. In order to reconstruct the KeraSkin™ model, cultured keratinocytes were transferred to the inner well of Millicell® (Millipore, USA) with a diameter of 12 mm, and cultivated on the air-liquid interface for 3 weeks to regenerate stratified 3D human epidermis. Quality Control (QC) of the KeraSkin™ model was performed by examining OD of negative control, ET_{50} with 1.0 % Triton-X100, IC_{50} with SDS (sodium dodecyl sulfate) and histological data. Details on QC procedure can be found in section VII Protocol and Appendix 3. For the transport of tissues, 24 tissues per kit are placed on the top of agarose gel and delivered at room temperature. Details of preparation and characterization of the KeraSkin™ model were published (Jung et al., 2014). The KeraSkin™ is a trademark registered in Korea. There is no IP issue for model manufacture, media, or test method.

V. The Structure of Validation Management Team

This validation study for the KeraSkin™ SIT was conducted with the support of Korean Ministry of Food and Drug Safety (MFDS). In this study, the validation management team organized by KoCVAM led the process of experiment design, the selection of test materials and the review and approval of the test protocol. Fig. 1 shows the VMT configuration and participating laboratories for this validation study.



[Figure 1] Organization of the KeraSkin™ SIT Validation Management Team

1. Validation Management Team (VMT)

The VMT was organized as shown in Fig.1. The VMT performed integrated management and scheduling of the entire validation process, selection and distribution of test materials, evaluation and statistical verification of test results, and drawing conclusions. The VMT also discussed and approved the changes to the protocol and other deviations from the original project plan.

2. Trial Coordinator

According to the agreement of the VMT members, Dr. Chun, Young Jin (Chung-Ang Univ.) was appointed as the VMT chairperson. The lead laboratory prepared a project plan, and VMT meetings were held with VMT members and representatives from lead laboratories. KoCVAM undertook the overall coordination work on this validation study and VMT meetings.

3. Chemical Selection, Acquisition, Coding, and Distribution

The Chemical Selection Team was responsible for selecting, listing, coding and supplying test chemicals. Dr. Kang, Mi-Jeong (Yeungnam University) and Ms. Lee, Gayoung (KoCVAM) took charge of the overall test chemical management and prepared for chemical selection report.

4. Data Analysis (Biostatistician) Group

A biostatistics team was organized as shown in Fig.1, and was responsible for data analysis and statistical verification. Dr. Bae, Seung Jin (Ewha Womans University) took charge of data analysis and statistical processing.

5. Lead Laboratories

The lead laboratories and participating laboratories were organized as shown in Fig. 1. Dr. Lim, Kyung-Min (Ewha Womans University), who was an original test method developer along with the AmorePacific R&D center, attended VMT meeting as the representative of the lead laboratory to present the introduction of the test method and progress of the validation study, and to respond to questions and comments of the VMT members. Yet, Dr. Lim was not involved in review opinions on the validation study and did not participate in the selection or coding of test materials. Biosolution Co. (Dr. Lee, Su Hyun) has joined the validation study as a co-lead laboratory since 2018.

6. Participating Laboratories

Biosolution Co. has acted as a participating laboratory as well as a co-lead laboratory. Korea Conformity Laboratories, COSMAX and Biotoxtech Co. have joined the validation study as participating laboratories.

7. Sponsorship

VMT members:

Chair	Chun, Young Jin (Chung-Ang Univ., College of Pharmacy)
Scientific advisory members	Son, Youngsook (Kyunghee Univ., College of Life Sciences) Kim, Bae-Hwan (Keimyung Univ., College of Natural Sciences) An, Susun (AmorePacific Co., R&D center) Yun, Jun Won (Catholic Univ., College of Biotechnology) Yoon, Hae Seong (KoCVAM)
Biostatistician	Bae, Seung Jin (Ewha Womans Univ., College of Pharmacy)
Assistant administrator	Kim, Tae Sung (KoCVAM)

Chemical manager	Kang, Mi-Jeong (Yeungnam Univ., College of Pharmacy) Lee, Ga Young (KoCVAM)
Lead laboratory	Lim, Kyung-Min (Ewha Womans Univ., College of Pharmacy) Lee, Su Hyun (Biosolution Co. Ltd.)

8. Laboratories

Laboratories that have participated in the validation study are listed below. Ewha Womans Univ., College of Pharmacy (Dr. Lim, Kyung-Min) served as the lead laboratory and evaluated the transferability, BLR, WLR, and predictive capacity of the test method. Biosolution Co. served as both lead laboratory as a model developer and participating laboratory. Korea Conformity Laboratories, COSMAX and Biotoxtech Co. joined as participating laboratories. The participating laboratories are geographically located apart each other across South Korea and not affiliated mutually.

Lead Laboratory 1 (Study director: Dr. Lim, Kyung-Min)

College of Pharmacy, Ewha Womans University,
52 Ewhayeodae-gil, Seodaemun-gu, Seoul, Republic of Korea

Lead Laboratory 2 & Participating Laboratory 1 (Study director: Dr. Lee, Su Hyun)

Biosolution Co., Ltd.
Room 519, Seoul Techno Park, 232 Gongneung-ro Nowon-Gu, Seoul, Republic of Korea

Participating Laboratory 2 (Study directors: Dr. Kim, Jin Sik & Dr. Kim, Ki Hun)

Korea Conformity Laboratories (KCL),
8 Gaetbeol-ro, 145 Beon-gil, Yeonsu-gu, Incheon, Republic of Korea

Participating Laboratory 3 (Study director: Ms. Chang, Yu Jin)

COSMAX Co.,
Building E, Pangyo Innovalley, 255 Pangyo-ro, Bundang-gu, Seongnam-si, Gyeonggi-do, Republic of Korea

Participating Laboratory 4* (Study director: Dr. Jung, Mi Sook)

Biotoxtech Co., Ltd.
Safety Evaluation Team
686-2 Yangcheong-ri, Ochang-eup, Cheongwon-gu, Cheongju-si, Chungbuk, Republic of Korea

* Note: Biotoxtech Co., Ltd. joined only transferability evaluation for validation trials.

Table 1 briefly shows the main roles of each laboratory that participated in this validation study.

[Table 1] Role of laboratories related to the validation study of KeraSkin™ SIT

	Technical Transfer		**Main Study**			**Supplementary Study**
	Technical Transfer-Trainer	Technical Transfer-Trainee	Lead laboratory	Proficiency	WLR/BLR for PS	Additional predictive tests*
Ewha Womans University	O		O			
Biosolution Co., Ltd	O		O	O	O	O
Korea Conformity Laboratories		O		O	O	O
COXMAX Co.		O		O	O	
Biotoxtech Co., Ltd.		O				

*In addition to the main predictive capacity test with the 20 reference chemicals, additional predictive tests were conducted with 46 test chemicals.

9. Timeline of KeraSkin™ SIT PS based validation study

Overall timeline of KeraSkin™ SIT PS based validation study is as shown below.

[Table 2] Timeline of KeraSkin™ SIT PS based validation study

Timelines	Task
2018. 04.	Validation study initiation
2018. 06.	○ 1st VMT meeting - Approval of participating laboratories and selection of test chemicals - Review and approval of validation study protocol (Protocol v. 1.4)
2018. 05. - 2018. 07.	Transferability test conducted in Biotoxtech Co.Ltd. and KCL
2018. 09. - 2018. 10.	1st-3rd within-/between-laboratory reproducibility test
2018. 11.	○ 2nd VMT meeting - Review of the revised protocol (Protocol v.1.5) - Approval of additional participating laboratory, COSMAX

2018. 12.	Transferability test conducted in COSMAX
2019. 01.	○ 3rd VMT meeting - Approval of the revised protocol (Protocol v.1.5)
2019. 01. - 2019. 03.	Predictive capacity test conducted in Biosolution Co. and KCL
2019. 03. - 2019. 05.	Predictive capacity test conducted in COSMAX
2019. 04.	○ 4st VMT meeting - Review of predictive capacity test results (Biosolution Co., KCL and COSMAX)
2019. 08.	○ 5st VMT meeting - Selection of additional predictive capacity test chemicals
2019. 08.	Predictive capacity test conducted in Biosolution Co. and KCL
2019. 10.	○ 6st VMT meeting - Review of predictive capacity test results (Biosolution Co. and KCL)
2019. 12.	○ 7st VMT meeting - Review and revise of the validation report

VI. Study Design

1. Comparison of the KeraSkin™ SIT and the Epiderm™ SIT (OECD TG 439)

The KeraSkin™ SIT is based on measuring the viability of cells that are exposed to test chemicals, and in principle it is similar to the Epiderm™ SIT which is a VRM of OECD TG 439. A comparison of test components for the KeraSkin™ SIT and the Epiderm™ SIT is given in Table 3.

[Table 3] Comparison of test components for the KeraSkin™ SIT and the Epiderm™ SIT

Test component (Required per performance standards for OECD TG 439)	KeraSkin™ SIT		Epiderm™ SIT[a]	
Cell source (Relevant human-derived cells)	Primary human keratinocytes		Primary human keratinocytes	
Pre-exposure	22 ± 2 hr incubation		Overnight (18 ± 3 hr)	
Tissue replicates (Min. of 2 tissues)	3 tissues		3 tissues	
Application of test chemicals	Liquid	Solid	Liquid	Solid

Quantity	40 µL (67 µL/cm^2)	D-PBS 40 µL + 40 mg (67 mg/cm^2)	30 µL (50 µL/cm^2)	D-PBS 25 µL + 25 mg (42 mg/cm^2)
Negative control	Dulbecco's phosphate buffered saline (D-PBS)		Dulbecco's phosphate buffered saline (D-PBS)	
Positive control	5% SDS[b] in DDW		5% SDS[b] in DDW	
Application period	30 ± 1 min		60 ± 1 min	
Washing	10 times of rinsing with a stream of DPBS		15 times of rinsing with a stream of DPBS and 3 times submerging in 150 mL DPBS	
Cotton swab	Yes		Yes	
Post-application period (Optimize as appropriate)	42 ± 2 hr		42 ± 2 hr (24 ± 2 hr & 18 ± 2 hr after replacing with fresh medium)	
Cell viability measurement	MTT assay		MTT assay	
Cell viability threshold value	50%		50%	

[a] Epiderm SIT protocol in MatTek website (https://www.mattek.com/wp-content/uploads/EPI-200-SIT-Skin-Irritation-Test-Protocol-MK-24-007-0023.pdf)
[b] SDS: sodium dodecyl sulfate

Cell viability is measured in the KeraSkin™ SIT using an MTT assay which is commonly adopted in other VRMs of TG 439.

The VMT considered the KeraSkin™ SIT to be a similar method to the RhE SIT described in OECD TG 439, and this validation study for the KeraSkin™ SIT was planned in accordance with the GD 220 performance standards (OECD, 2015a).

2. Test Chemicals

2.1 Chemical Selection

As listed in Table 4, the selected testing chemicals are the 20 reference chemicals listed in the OECD GD 220 performance standards.

[Table 4] List of the 20 reference chemicals used in the KeraSkin™ SIT validation study

Non-Irritant (NI)				Irritant (I)			
No.	**Chemical name**	**CAS**	**UN GHS**	**No.**	**Chemical name**	**CAS**	**UN GHS**
1	1-Bromo-4-chlorobutane	6940-78-9	No Cat.	**11**	1-Decanol	112-30-1	Cat. 2

2	Diethyl phthalate	84-66-2	No Cat.	**12**	Cyclamen aldehyde	103-95-7	Cat. 2
3	Naphthalene acetic acid	86-87-3	No Cat.	**13**	1-Bromohexane	111-25-1	Cat. 2
4	Allyl phenoxy-acetate	7493-74-5	No Cat.	**14**	2-Chloromethyl-3,5-dimethyl-4-methoxypyridine HCl	86604-75-3	Cat. 2
5	Isopropanol	67-63-0	No Cat.	**15**	Di-n-propyl disulphide	629-19-6	Cat. 2
6	4-Methyl-thio-benzaldehyde	3446-89-7	No Cat.	**16**	Potassium hydroxide (5% aq.)	1310-58-3	Cat. 2
7	Methyl stearate	112-61-8	No Cat.	**17**	Benzenethiol, 5-(1,1-dimethylethyl)-2-methyl	7340-90-1	Cat. 2
8	Heptyl butyrate	5870-93-9	No Cat. (Optional Cat. 3)	**18**	1-Methyl-3-phenyl-1-piperazine	5271-27-2	Cat. 2
9	Hexyl salicylate	6259-76-3	No Cat. (Optional Cat. 3)	**19**	Heptanal	111-71-7	Cat. 2
10	Cinnamaldehyde	104-55-2	No Cat. (Optional Cat. 3)	**20**	Tetrachloroethylene	127-18-4	Cat. 2

2.2 Coding and Distribution

Test chemicals were selected with the guidance of the VMT. The test chemicals were encoded (with different coding for each of 3 test runs) and provided separately to each participating laboratory for each run by KoCVAM. Coding information was included in Appendix 2.

3. Study Quality Criteria

The study was conducted in compliance with GLP principles. Two laboratories (Biotoxtech and Korea Conformity Laboratories [KCL]) are OECD GLP-accredited contract research organizations in Korea. Biosolution Co. and COSMAX conducted the tests in GLP spirit. QA sheets at every run were checked by KoCVAM to ensure the quality of the study. QAU documents are provided in Appendix 6.

4. Defined Reliability and Accuracy Value

4.1 Transferability of the Test Method

Three participating labs (KCL, Biotoxtech and COSMAX) were trained for the test method by Biosolution Co. as lead laboratory, based on the protocol 1.4.

Transferability was checked with 6 chemicals for KCL and Biotoxtech, and 4 chemicals for COSMAX as shown in Table 5.

[Table 5] Results of transferability tests in three participating labs: KCL, Biotoxtech, and COSMAX

	Chemical	CAS No.	State	GHS	VRM	KeraSkin™ Viability (%)	Irritancy	Pass/Fail
Korea Conformity Laboratories (KCL)	Methyl stearate	**112-61-8**	Solid	No Cat.	NI	104.79 ± 2.63	NI	**Pass**
	Heptyl butyrate	**5870-93-9**	Liquid	No Cat. (Optional Cat. 3)	NI	102.34 ± 0.32	NI	**Pass**
	Hexyl salicylate	**6259-76-3**	Liquid	No Cat. (Optional Cat. 3)	NI	102.29 ± 2.84	NI	**Pass**
	1-Decanol	**112-30-1**	Liquid	Cat. 2	NI	30.58 ± 3.27	I	**Pass**
	2-Chloromethyl-3,5dimethyl-4-methoxy pyridine-HCI	**86604-75-3**	Solid	Cat. 2	I	2.13 ± 0.12	I	**Pass**
	5% Potassium hydroxide	**1310-58-3**	Liquid	Cat. 2	I	0.05± 0.23	I	**Pass**
Biotoxtech	Methyl stearate	**112-61-8**	Solid	No Cat.	NI	93.24± 6.08	NI	**Pass**
	Heptyl butyrate	**5870-93-9**	Liquid	No Cat. (Optional Cat. 3)	NI	69.96 ± 7.34	NI	**Pass**
	Hexyl salicylate	**6259-76-3**	Liquid	No Cat. (Optional Cat. 3)	NI	91.14 ± 3.59	NI	**Pass**
	1-Decanol	**112-30-1**	Liquid	Cat. 2	NI	6.35 ± 0.30	I	**Pass**
	2-Chloromethyl-3,5dimethyl-4-methoxy pyridine-HCI	**86604-75-3**	Solid	Cat. 2	I	2.14 ± 0.07	I	**Pass**
	5% Potassium hydroxide	**1310-58-3**	Liquid	Cat 2	I	2.14 ± 1.05	I	**Pass**
COSMAX	Methyl stearate	**112-61-8**	Solid	No Cat.	NI	85.62 ± 3.44	NI	**Pass**
	Hexyl	**6259-**	Liquid	No Cat.	NI	78.54 ± 9.19	NI	**Pass**

	salicylate	**76-3**		(Optional Cat. 3)				
	2-Chloromethyl-3,5dimethyl-4-methoxy pyridine-HCI	**86604-75-3**	Solid	Cat. 2	I	2.93 ± 0.36	I	**Pass**
	5% Potassium hydroxide	**1310-58-3**	Liquid	Cat 2	I	-0.79 ± 0.70	I	**Pass**

* Viability values are mean ± SD of ≥ 3 wells. Detailed information on the technical transferability is available in Appendix 4.

4.2 Within-Laboratory Reproducibility (WLR)

The OECD GD 220 performance standards advise that the RhE SIT similar to TG 439 should meet the within-laboratory reproducibility of equal to or higher than 90%. A total of 3 runs were conducted for each of the 20 reference chemicals by each participating laboratory. All 3 laboratories completed runs of 100%. The ratio of concordance of decisions within 3 runs was calculated as percent of reproducibility. The results of WLR are presented in section VIII.

4.3 Between-Laboratory Reproducibility (BLR)

The OECD GD 220 performance standards advise that the RhE SIT similar to TG 439 should meet the between-laboratory reproducibility of equal to or higher than 80%. A total of 3 runs were conducted for each of the 20 reference chemicals by each participating laboratory. And the ratio of concordance of decisions made from the average viability of each laboratory was calculated as percent of reproducibility. The results of BLR are presented in section VIII.

4.4 Predictive Capacity

The OECD GD 220 performance standards describe that the acceptable criteria for predictive capacity is equal to or better than 80% sensitivity, 70% specificity and 75% accuracy.

To evaluate the predictive capacity of KeraSkin™ SIT, the 20 reference chemicals in the OECD GD 220 performance standards were tested three times by each participating laboratory. Nine runs were carried out for each chemical (3 runs by 3 laboratories), which can be summed up to 180 runs in total. The predictive capacity was calculated in the way that 180 runs were estimated independently.

Additionally, the data for 46 test chemicals (chemicals tested to evaluate predictive capacity of KeraSkin™ SIT), which were selected from previous validation study report and scientific papers, with known *in vivo* irritancy, composed of 27 liquids and 19 solids (4 corrosives and 7 irritants and 35 non-irritants) were evaluated according to the protocol 1.5 to assess the predictive capacity of KeraSkin™ SIT. The agreement of binary decisions of irritant or non-irritant with *in vivo* classification was assessed. Predictive capacity was presented as sensitivity, specificity, and accuracy. For each chemical with 3 runs, an arithmetic mean value was obtained and used for the decision. The results of the predictive capacity are presented in section VIII. Predictive capacity was also assessed with a weighted approach

and its results are presented in section IX.

5. Data Collection, Handling, and Analysis

The Lead laboratory, Ewha Womans Univ., College of Pharmacy (Dr. Lim, Kyung-Min) collected data sheets from participating laboratories in coded status at the end of each run, tabulated data results and handed them over to KoCVAM. Then, KoCVAM provided the coding information for test chemicals after checking the validity of the data. A final data was analyzed by biostatisticians. The results were reported to, and approved by the VMT.

VII. Protocol

A protocol for the KeraSkin™ SIT was developed by the AmorePacific R&D center and enacted as ver. 1.0 on 16th April, 2012. The protocol was optimized through 5 times of revisions and finalized as version 1.5. Overview of the protocol revision and the chemicals used during protocol optimization are as follows.

[Table 6] Overview of the KeraSkin™ SIT protocol revision and chemicals used for optimization

Version (approval date)	**Revision**	**Chemicals used for optimization**
Protocol 1.0 (Apr 16th 2012)		20% SLS
Protocol 1.1 (Apr 8th 2013)	Addition of the MTT and Nylon Mesh reactivity assay for test chemicals	20% SLS
Protocol 1.2 (Sep 7th 2015)	Changes in the washing procedure - 10 mL overflowing, mesh removed - Rinse outside of the insert - Remove DPBS using sterile gauze and cotton swabs	1,5-Hexadiene (CAS RN 592-42-7) 3,3-Dimethylpentane (CAS RN 562-49-2) Isopropanol (CAS RN 67-63-0)
Protocol 1.3 (Dec 1st 2016)	Addition of frozen tissue production method and interference test flow chart Changes in the washing procedure - Rinse inside of the insert 10 times using poly wash bottle	1-bromobutane (CAS RN 109-65-9) 1-bromopentane (CAS RN 110-53-2) 1-bromohexane (CAS RN 111-25-1) Allyl phenoxy acetate (CAS RN 7493-74-5) Heptyl butyrate (CAS RN 5870-93-9)
Protocol 1.4 (Jun 5th 2018)	Changes in the QC criteria in test laboratory - OD value of NC: $0.7 \leq OD_{NC} \leq 1.6$ - CV% of PC: ≤ 40% Change MTT concentration: 0.3 mg /mL → 0.4 mg/mL Changes in the application procedure of test chemicals - Change to mesh-free when applying test material - Changes in the amount of the	A-terpineol (CAS RN 98-55-5) 1-bromopentane (CAS RN 110-53-2) Butyl methacrylate (CAS RN 97-88-1) Capric acid (decanoic acid) (CAS RN 334-48-5) Allyl phenoxy acetate (CAS RN 7493-74-5) 1-bromohexane (CAS RN 111-25-1) Di-n-propyldisulphide (CAS RN 629-19-6) 3,3-Dithiodipropionic Acid (CAS RN 1119-62-6) 4-Amino-1,2,4-Triazole (CAS RN 584-13-4)

	treating material: 30 μL → 40 μL (solid: 30 mg → 40 mg) to compensate the exclusion of mesh - Duration of the treatment: 45 min → 30 min - Optimize to correct false prediction Addition of re-test criteria	Erucamide (CAS RN 112-84-5) Benzyl Salicylate (CAS RN 118-58-1) Sodium Bisulphite (CAS RN 7631-90-5)
Protocol 1.5 (Jan 11th 2019)	Additions in washing step	Naphthalene acetic acid (CAS RN 86-87-3) 3,3-Dithiodipropionic Acid (CAS RN 1119-62-6) 4-Amino-1,2,4-Triazole (CAS RN 584-13-4) Erucamide (CAS RN 112-84-5) 1,5-hexadiene (CAS RN 592-42-7) Allyl phenoxy acetate (CAS RN 7493-74-5) Polyethylene glycol 400 (CAS RN 25322-68-3) 3,3-Dimethylpentane (CAS RN 562-49-2) α-Terpineol (CAS RN 98-55-5) 1-Bromopentane (CAS RN 110-53-2) Butyl methacrylate (CAS RN 97-88-1)

*Underlined chemicals: OECD TG439 PS chemicals

During the protocol revision, some PS chemicals were used for optimization. Detailed protocol and revision history are included in Appendix 3. Major points of protocol optimization were as follow;

- *Removal of mesh and change of application volume and time: Viscous substance sometimes made the mesh stick to the tissue even after the washing process, so the mesh was removed. Instead the treatment volume was increased (30 μL → 40 μL) to evenly distribute the substance on the tissue and the treatment time (45 min → 30 min) was shortened to compensate the increased treatment volume. Post-incubation time was set to 42 hr as the existing VRMs of OECD TG 439.*

- *Prediction model: An irritant is predicted if the mean relative tissue viability of minimum three replicate tissues exposed to the test chemical is reduced to 50% or below of the mean viability of the negative controls. Since 50% cutoff value is adopted by all existing VRMs of OECD TG 439, KeraSkin™ SIT has been optimized to 50% cutoff.*

- *Acceptance criteria: Acceptance criteria for KeraSkin™ SIT were established referring to existing VRMs of OECD TG 439.*

The main validation data (i.e., WLR, BLR and predictive capacity based on the 20 reference chemicals) and supplementary data for additional 46 chemicals were generated according to protocol ver. 1.5.

1. Protocol for the KeraSkin™ SIT

Quality Control (QC) of the KeraSkin™ SIT model is performed for each batch by examining OD value, ET_{50} with 1.0 % Triton X-100, IC_{50} with SDS (sodium dodecyl sulfate), and histological data. To assess tissue viability for QC, a total of 24 KeraSkin™ tissues were used. The tissues are placed in the wells of 24-well plates containing 0.3 mL of MTT medium (0.5 mg/mL; Sigma-Aldrich, MO, USA) and are incubated for 3 hr (37°C, 5% CO_2 condition). Formazan produced in the tissues is extracted with isopropanol (2 mL). The optical density (OD) of the extract (250 μL) is measured at 570 nm and

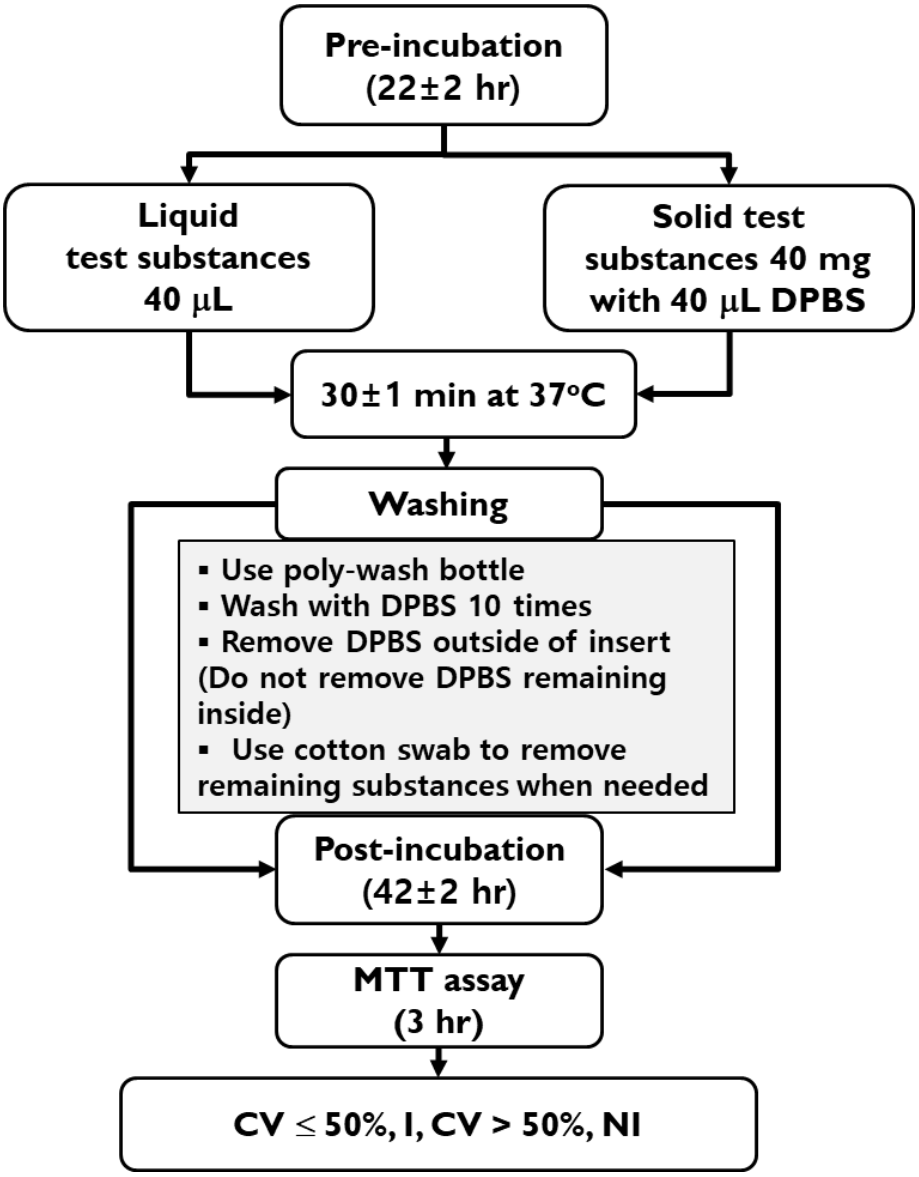
at 650 nm as a reference absorbance. Isopropanol is used as a blank.

To determine ET_{50}, a solution of 1 % Triton X-100 (50 µL) is applied to the KeraSkin™ and left for various lengths of time (0, 3, 5, 7, and 22 hr) referring to a previous published method (MatTeK 2002). Then, the cell viability is measured with an MTT assay. Using a semi-log scale, plot the % viability (linear y axis) versus the dosing time (log x axis). By interpolation, the time at which the % viability has dropped to 50% is considered the ET_{50} value.

To check for IC_{50}, various concentrations (0, 2, 3, 4, 5, and 6 mg/mL) of SDS (50 µL) is applied to the KeraSkin™ for 18 hr. Then, the cell viability is measured with an MTT assay. Using a semi-log scale, plot the % viability (linear y axis) versus the concentration (log x axis). By interpolation, the concentration at which the % viability has dropped to 50% is considered the IC_{50} value.

It should be noted that the MTT assay condition for QC is different from that of KeraSkin™ SIT, which uses 0.3 mL of 0.4 mg/mL MTT solution. The MTT assay condition for QC was established referring to existing VRMs and manufacturer's instructions. Details on QC procedure can be found in Appendix 3 and Appendix 8, and QC results were provided in QC report (Appendix 8).

The KeraSkin™ model is delivered to the testing labs in a 24-well format on agarose gel at room temperature. The quality control is conducted with OD values of negative control: 0.6-1.2, ET_{50}: 6.9-14.0 hr, IC_{50} for SDS 1.5-4.8 mg/mL and histological examination. Upon receipt of the shipment, culture medium is pre-warmed in a 37°C thermostat for 30 min. As preparation for the pre-incubation step, 900 µL of the pre-warmed medium is added to each well of a 6-well plate using micropipette and the RhE model insert is carefully transferred to the wells using forceps. Then the well-plate is pre-incubated at 37°C, 5% CO_2 for 22±2 hr. To conduct the experiment, test substances (40 µL of liquid substance or 40 mg of solid substance in 40 µL DPBS) are topically applied to the upper epithelial surface of the model insert after the pre-incubation. The tissue is incubated (37°C, 5% CO_2 condition) again for 30 ± 1 min (Fig. 2). Then the tissue is washed to remove the test substances as described in Fig. 2 and further incubated (37°C, 5% CO_2 condition) for 42 ± 2 hr. The resulting tissue viability is determined by MTT assay.

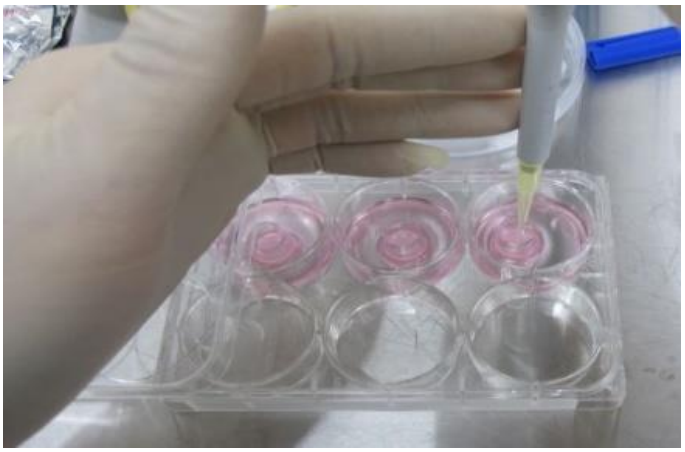


[Figure 2] Overview of the KeraSkin™ skin irritation test

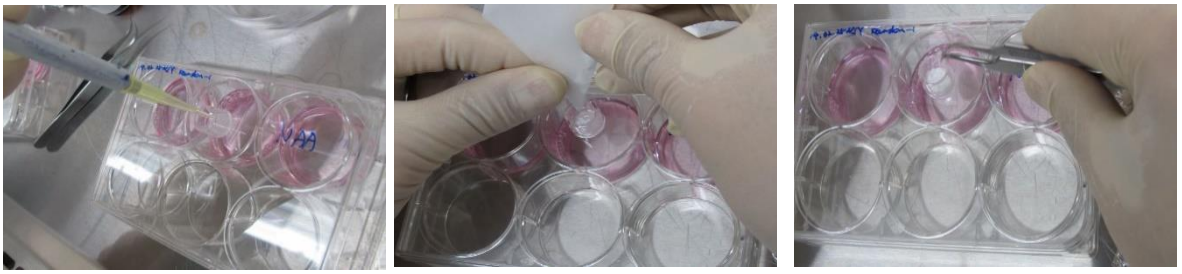
1.1 KeraSkin™ SIT Protocol (version 1.5)

- Application of the test chemical:

  Liquid test substances should be applied under the chemical hood since there are many chemicals that are volatile and odorous. Take 40 μL of test material with a micropipette and slowly drop it atop and at the center of the RhE model (Fig. 3). For solid test substances, the tissue surface should be moistened with 40 μL DPBS and then 40 mg of test substance is applied atop and at the center of the RhE model (Fig. 4). For both liquid and solid substances, after application, swirl the insert uniformly to spread the substance evenly over the entire surface. When application of test chemicals in 6-well units is done, use the plate cover film to prevent evaporation and volatilization, and put the plate in a 5% CO_2 incubator at 37°C for 30 ± 1 min.

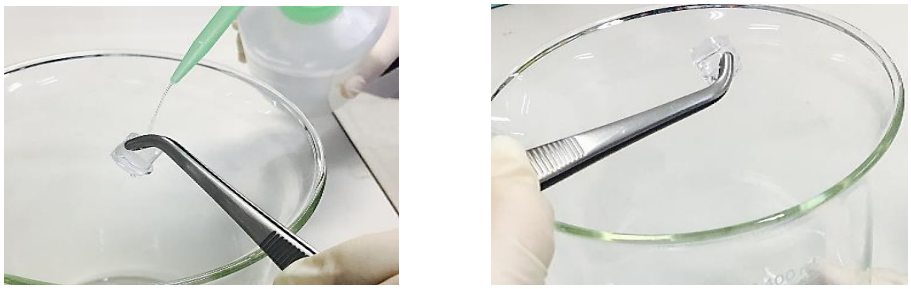


[Figure 3] Application of liquid substances on tissues; With an autopipette, dispense 40 μL of liquid test substance directly atop the tissue and spread gently by swirling.



[Figure 4] Application of solid substances on tissues; First, apply 40 μL of DPBS for wetting the surface of the insert (left), apply the weighed test chemical to the insert (center) and then swirl the insert to spread evenly (right).

- Washing: Washing process of the 6-wells should be done within 20 min. DPBS for washing should be kept warm in a 37°C thermostat before use. Remove the treated tissue from the incubator, flow the DPBS to side well of the insert using a poly wash bottle, and when the DPBS is fully filled in the insert, flip the insert over the beaker at once (Fig. 5). At this time, be careful not to touch the beaker with forceps because it might damage the tissue inside the insert.



[Figure 5] Washing with DPBS using a squeeze bottle (left) and flipping of inserts to remove DPBS (right)

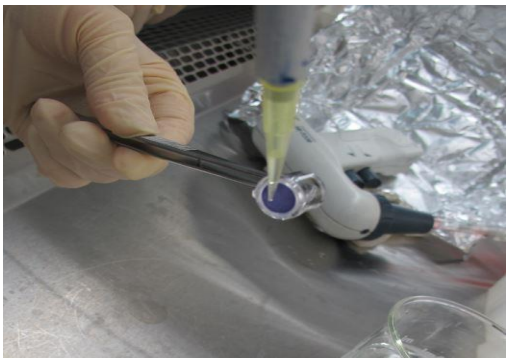
- Repeat the step above 9 times to wash the tissue for 10 times in total. However, if considerable amount of residual material (i.e. oils or highly viscous liquid materials or solid materials which react with washing liquid to cause gelation) is still observed after 5 washes, wipe the residuals with a cotton swab.

- Remove the DPBS outside of the insert using sterile gauze after washing (Fig. 6). However, do not remove the residual DPBS inside the insert.

[Figure 6] Removal of DPBS from outside of the insert

- Post-incubation: Pre-warm the 6-well plate, pre-filled with 0.9 mL of culture of medium by placing in a 37℃, 5 % CO_2 incubator. Transfer the washed tissue to a prepared 6-well plate and incubate for 42±2 hr in a 37℃, 5% CO_2 incubator.

- MTT assay: After post-incubation, remove the media from both inside and outside of the insert using a pipette. Transfer inserts into 24-well plates, prefilled with 200 μL of 0.4 mg/mL MTT per well. Add 100 μL of MTT solution inside the insert. Cover the plate with aluminum foil for protecting from the light and place in the incubator (37℃, 5% CO_2) for 3 hr ± 5 min. Remove MTT residual from the inside/outside of the insert using 200 μL pipette when MTT treatment is done.



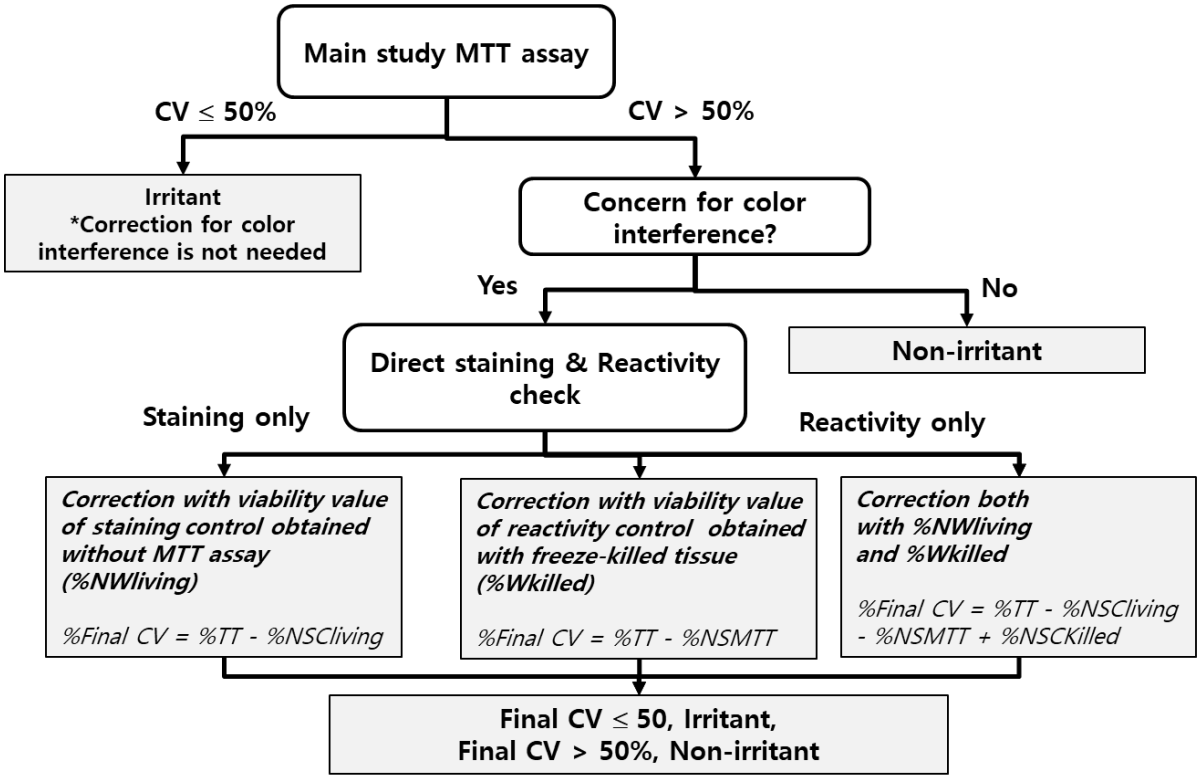
[Figure 7] Removal of MTT from inside/outside of the insert after MTT treatment

- Transfer the insert to a new 6-well plate containing 1.9 mL/well of isopropanol, and then apply 100 μL of isopropanol to the inside of the insert. Apply plate cover film to the isopropanol-treated 6-well plates, protect from the light using aluminum foil, put in a zipper bag and place it on a shaker for 3 hr ± 5 min to extract formazan.

    - Optical Density Measurements: After formazan extraction, combine the extract from inside and outside of the insert, remove the tissue, and mix well by pipetting enough such that formazan crystals are not seen in each well. Transfer 250 μL of extract per well into a 96 well plate, and measure the optical density (OD) using a 96 well plate spectrophotometer (wavelength: 570 nm). Take mean value of three wells as a final value.

1.2 Detecting and Correcting Interference from Coloured Chemicals with MTT Endpoints

In order to prevent the possible interference with the MTT endpoint from coloured chemicals, ‘direct staining by test materials’ or ‘MTT reactivity’ may be checked. Though, when a test substance is determined to be an irritant, the correction is not necessary since correction always over-predict the classification of skin irritancy by subtracting the cell viability. Accordingly, the colour interference corrections are reserved as optional for the test chemicals with significant concerns. Overall scheme for correcting colour interference is as follows.



[Figure 8] Overall flow-chart for correcting colour interference

Checking for direct staining

Coloured test substance may interfere with OD measurement of MTT formazan by directly staining the tissue.

Add 0.5 mL of deionized water and 40 μL (liquid) or 40 mg (solid) of the test chemical to each Eppendorf tube and incubate the mixture in a cell incubator (37°C, 5% CO_2, 95% RH) for 30 min. Observe the colour changes, and perform a functional check with RhE tissue if there is significant colour change.

Checking for MTT reactivity

Test substance with reducing potential may reduce MTT directly and form formazan by itself.

Add 0.5 mL of MTT solution (0.4 mg/mL) and 40 μL (liquid) or 40 mg (solid) of the test chemical to each Eppendorf tube and incubate the mixture in a cell incubator (37°C, 5% CO_2, 95% RH) for 1 hr. Observe the colour change and if the MTT solution changes to blue/purple, perform a functional check with freeze-killed tissues. Correction of the cell viability may be necessary as explained below.

Correction of cell viability for the interference of MTT assay

1) When the test substance is only colourant;

After applying 40 μL or 40 mg of the test substance to the living tissue surface, wash it in the same manner as described in the washing step of this test method. However, in the MTT assay, only

phenol red free-DMEM without MTT is added and cultured for 3 hr. The NSCLiving control is evaluated using living tissue. Living tissues may possess inherent biological variability between batches, and this necessitates a calibration test for each run. The final cell viability is obtained via cell viability before correction (TT%) minus the cell viability from this test.

$$\%Final\ CV = \%TT - \%NSCliving$$

2) When the test substance is non-colourant, but MTT reactive;

Conduct the test according to the main protocol but use a freeze-killed tissue (NSMTT [Non-specific MTT reduction control]) instead of a viable tissue. The freeze-killed tissue has no metabolic activity, but is capable of absorbing and binding to the test substance. Upon receipt of the tissue, the tissue must be frozen in a 24-well plate at -80℃ for 48 hr. Then transfer the tissue into a 6-well plate containing 0.9 mL fresh medium and allow 10 min for stabilization. This functional check is not done for every test run, but can be done once, in duplicate, for each test substance of concern. However, if the staining ratio is less than 5%, correction is not necessary. If the staining ratio is 30% or more, it cannot be compatible with this test. If the cell viability is 50% or less, it is determined to be an irritant. The resulting cell viability (%NSMTT) is used to correct cell viability as follows,

$$\%Final\ CV = \%TT - \%NSMTT$$

3) When the test substance is both colourant and MTT reducer;

In this case, the correction needs to be done for both colourant and MTT reducer as described in 1) and 2), but the colour interference will be subtracted twice. To achieve this, another functional check with freeze-killed tissue must be conducted with phenol red free-DMEM instead of MTT solution and culture for 3 hr. Prepare freeze-killed tissue as described in 2), and conduct the test once, in duplicate, for each test substance. Final cell viability is calculated as follows,

$$\%Final\ CV = \%TT - \%NSCliving - \%NSMTT + \%NSCKilled$$

2. Prediction Model for the KeraSkin™ SIT

According to the EU and the GHS classification, an irritant is predicted if the mean relative tissue viability of minimum of three individual tissues exposed to the test chemical is reduced to 50% or below of the mean viability of the negative controls. The test chemical was defined as a non-irritant if the tissue viability was higher than 50% (Table 7).

[Table 7] Prediction model for the KeraSkin™ SIT

Prediction model	Classification	UN GHS Category
Mean tissue viability is ≤ 50%	Irritant (I)	Category 2*
Mean tissue viability is > 50%	Non-Irritant (NI)	No Category

*provided that *in vitro* skin corrosion test was negative

3. Acceptance Criteria

3.1 Negative Control

The negative control chemical is DPBS, and the OD value of the negative control chemical should be 0.7 or more and 1.6 or less.

$$0.7 \leq \text{Mean OD measured value} \leq 1.6$$

3.2 Positive Control

The positive control material should be 5% SDS and the average cell viability of positive control should be 40% or less.

$$\text{Mean tissue viability of positive control} \leq 40\%$$

3.3 Criteria for a Re-test

A test is regarded as acceptable as long as results do not fall into the criteria for a re-test shown below.

- Criteria for a re-test

1) If absorbance value of the negative control chemical is less than 0.7 or exceeds 1.6.
2) If a cell viability value of the positive control chemical is more than 40%.
3) In negative control chemical, positive control chemical, and test chemical: When the standard deviation of the cell viability value of treated tissue in replications exceeds 18%.
4) If the average cell survival rate of the chemical-treated-well is between 45% and 55%. (≥ 45% and ≤ 55%, borderline chemicals)
Note: If the re-test gives borderline value again, the concordance of the decisions between the original and re-test should be considered. If the original and re-test produced concordant decision, the decision is taken and additional re-test is not necessary. If the decisions differ, then the third test (2^{nd} re-test) should be conducted and the majority decisions of three tests should be taken.

3.4 Standard Deviation between Replicate Wells

Since skin irritation potential is predicted from the mean viability of tissues, the variability of tissue replicates must be kept at an acceptably low level. When three replicate tissues are used, the standard deviation (SD) of tissue viability of three identically treated replicates for the negative control, the positive control, and test chemicals should be 18% or less.

VIII. Results

1. QC of the Tissue Models

To ensure the quality of a manufactured KeraSkin^{TM} model, each batch was tested for the cell viability of the negative control (with respect to OD with MTT assay) and ET_{50} (an exposure time estimated to reduce the cell viability to 50% with a non-ionic detergent, Triton X-100 at the

concentration of 1.0% as measured with MTT assay). In addition, IC_{50} values for SDS and histological examination were conducted. The quality control tests were conducted with the tissue stored in a condition similar to that for tissue delivered to the test site (at 4ºC for at least 3 hr and pre-incubated overnight). Actual QC data for the 13 batches used for the main reproducibility study was as follows; NC OD: 0.9 ± 0.1, ET_{50}: 10.7 ± 1.5 hr, and IC_{50}: 3.3 ± 0.4 mg/mL (mean ± SD). QC tests have been done for all batches used in the validation study as reported in Appendix 8.

2. Quality Assurance

Assays and quality assurance were carried out in the spirit of GLP, although not all participating laboratories were GLP certified. The participating laboratories conducted the experiments according to Protocol 1.5. Essential steps of the test were checked during the conduct of the test. All raw data and data sheets were reviewed at each laboratory and then circulated to the VMT for checking errors and omissions. Independent QA confirmed that the results presented accurately reflect the raw data (Appendix 6).

3. Negative Control

Table 8 shows absorbance values for the negative control for 3 runs by three laboratories. It includes re-test runs which were required by the occurrence of invalid runs according to re-test criteria (due to re-test criteria #3 and #4). Twenty reference chemicals were divided into 2 parts for the test conduct, due to a load of participating laboratories and test procedures were the same for all parts. All data satisfied the acceptance criteria for the negative control, 0.7 ≤ mean OD ≤ 1.6 (actual values, 0.804-1.189).

[Table 8] Absorbance values for the negative control for 3 runs by three laboratories

	Lab 1*			**Lab 2****			**Lab 3*****		
	1	2	3	1	2	3	1	2	3
Part 1	0.804	0.804	0.882	0.883	1.045	1.016	0.918	1.189	0.878
Part 2	0.997	0.933	0.887	1.302	1.090	1.252	1.002	0.868	1.069
Re-test****	-	-	-	-	-	-	-	0.843	

* Lab 1: Biosolution Co., **Lab 2: Korea Conformity Laboratories, ***Lab 3: COSMAX
****Re-test was done due to re-test criteria #3 or criteria #4

4. Positive Control

Table 9 shows cell viabilities for positive control (5% SDS) for 3 runs by three laboratories. Twenty reference chemicals were tested. All data satisfied the acceptance criteria for positive control, and mean cell viability was ≤ 40%.

[Table 9] Cell viabilities (%) for the positive control for 3 runs by three laboratories

	Lab 1*			Lab 2**			Lab 3***		
	1	2	3	1	2	3	1	2	3
1st	3.00	5.37	4.51	5.04	1.60	1.73	4.36	2.83	21.09
2nd	3.16	2.67	5.33	5.97	3.96	3.41	2.94	2.07	2.34
Re-test****	-	-	-	-	-	-	-	1.75	

* Lab 1: Biosolution Co., **Lab 2: Korea Conformity Laboratories, ***Lab 3: COSMAX
****Re-test was done due to re-test criteria #3 or criteria #4

5. Reliability

5.1 Within-Laboratory Reproducibility (WLR)

WLRs at the three laboratories (Lab 1, Lab 2 and Lab 3) were evaluated for the 20 reference chemicals in the performance standards (Table 10). According to the OECD GD 220 PS, WLR was evaluated by estimating concordance in classifications (Irritant or Non-irritant) for three runs for each laboratory. As shown in Table 10, WLRs were 95% to 100%, which met the PS criteria of WLR ≥ 90%.

[Table 10] Within-laboratory reproducibility for the 20 reference chemicals

		Concordance (%)	PS	Pass/Failure for WLR
Lab 1*	Run 1~3	100%	≥ 90%	Pass
Lab 2**	Run 1~3	100%	≥ 90%	Pass
Lab 3***	Run 1~3	95%	≥ 90%	Pass

* Lab 1: Biosolution Co., **Lab 2: Korea Conformity Laboratories, ***Lab 3: COSMAX

5.2 Between-Laboratory Reproducibility (BLR)

After WLR tests, BLR between the three laboratories that had passed WLR criteria was evaluated for concordance in the classification of the 20 reference chemicals in the performance standards (Table 11). According to the OECD GD 220 PS, BLR was evaluated with the 20 reference chemicals by estimating concordance in the classification (Irritant or Non-irritant) made with the averaged viability value from each laboratory. As shown in Table 11, BLR was 100%, which met the PS criteria of BLR ≥ 80%.

[Table 11] Between-laboratory reproducibility for the 20 reference chemicals

No.	Chemical	Cas No.	Physical state	UN GHS	Final prediction (1-3)			Concordance
					Lab 1	Lab 2	Lab 3	
1	1-Decanol	112-30-1	liquid	Cat 2	TP	TP	TP	Y
2	Cyclamen aldehyde	103-95-7	liquid	Cat 2	TP	TP	TP	Y

3	1-Bromohexane	111-25-1	liquid	Cat 2	TP	TP	TP	Y
4	2-Chloromethyl-3,5-dimethyl-4-methoxypyridine HCl	86604-75-3	solid	Cat 2	TP	TP	TP	Y
5	Di-n-propyl disulphide	629-19-6	liquid	Cat 2	TP	TP	TP	Y
6	Potassium hydroxide(5% aq.)	1310-58-3	liquid	Cat 2	TP	TP	TP	Y
7	Benzenethiol, 5-(1,1-dimethylethyl)-2-methyl	7340-90-1	liquid	Cat 2	TP	TP	TP	Y
8	1-Methyl-3-phenyl-1-piperazine	5271-27-2	solid	Cat 2	TP	TP	TP	Y
9	Heptanal	111-71-7	liquid	Cat 2	TP	TP	TP	Y
10	Tetrachloroethylene	127-18-4	liquid	Cat 2	TP	TP	TP	Y
11	1-Bromo-4-chlorobutane	6940-78-9	liquid	NC	FP	FP	FP	Y
12	Diethyl phthalate	84-66-2	liquid	NC	TN	TN	TN	Y
13	Naphthalene acetic acid	86-87-3	solid	NC	TN	TN	TN	Y
14	Allyl phenoxy-acetate	7493-74-5	liquid	NC	TN	TN	TN	Y
15	Isopropanol	67-63-0	liquid	NC	TN	TN	TN	Y
16	4-Methyl-thio-benzaldehyde	3446-89-7	liquid	NC	FP	FP	FP	Y
17	Methyl stearate	112-61-8	solid	NC	TN	TN	TN	Y
18	Heptyl butyrate	5870-93-9	liquid	NC	TN	TN	TN	Y
19	Hexyl salicylate	6259-76-3	liquid	NC	TN	TN	TN	Y
20	Cinnamaldehyde	104-55-2	liquid	NC	FP	FP	FP	Y
					Between-Laboratory Reproducibility			**100% (20/20)**

TP: True positive, TN: True negative, FP: False positive, FN: False negative
Lab 1: Biosolution Co., Lab 2: Korea Conformity Laboratories, Lab 3: COSMAX

6. Predictive Capacity

6.1 Predictive Capacity for the 20 Reference Chemicals

The 20 reference chemicals from the performance standards of OECD GD 220 were evaluated by three laboratories by three runs independently, which led to a total of 180 determinations as shown below (Table 12). The test results were corrected for colour interferences.

[Table 12] Individual test results (% of a cell viability) for the 20 reference chemicals conducted three

times by three participating laboratories

No.	Chemical	Cas No.	Physical state	UN GHS	Lab 1			Lab 2			Lab 3		
					1	2	3	1	2	3	1	2	3
1	1-Decanol	112-30-1	liquid	Cat 2	8.94	2.28	11.26	8.56	3.21	10.18	12.18	13.48	13.69
2	Cyclamen aldehyde	103-95-7	liquid	Cat 2	2.14	0.10	5.01	4.01	1.10	2.62	2.41	-5.85	3.68
3	1-Bromohexane	111-25-1	liquid	Cat 2	18.58	4.77	7.10	10.11	8.23	13.07	41.57	33.80	33.18
4	2-Chloromethyl-3,5-dimethyl-4-methoxypyridine HCl	86604-75-3	solid	Cat 2	8.03	5.46	6.74	1.91	1.85	2.47	2.20	2.04	2.94
5	Di-n-propyl disulphide	629-19-6	liquid	Cat 2	24.60	24.69	27.50	2.45	8.22	14.39	38.18	38.22	62.51
6	Potassium hydroxide (5% aq.)	1310-58-3	liquid	Cat 2	1.80	-2.50	-1.17	0.43	-0.25	-0.95	-3.85	-4.48	0.36
7	Benzenethiol, 5-(1,1-dimethylethyl)-2-methyl	7340-90-1	liquid	Cat 2	7.33	6.53	5.66	-5.86	-11.12	0.62	-1.17	-4.46	20.93
8	1-Methyl-3-phenyl-1-piperazine	5271-27-2	solid	Cat 2	-0.95	1.21	4.16	0.27	4.46	1.55	9.35	30.84	-1.86
9	Heptanal	111-71-7	liquid	Cat 2	8.41	4.36	2.61	1.32	-0.85	2.70	-1.29	4.21	3.72
10	Tetrachloroethylene	127-18-4	liquid	Cat 2	9.45	2.94	7.56	7.10	6.09	6.66	39.74	12.59	34.70
11	1-Bromo-4-chlorobutane	6940-78-9	liquid	NC	15.85	20.97	20.35	5.92	2.90	9.31	3.38	4.54	23.57
12	Diethyl phthalate	84-66-2	liquid	NC	82.13	74.96	88.58	94.33	91.62	82.17	74.06	101.78	103.28
13	Naphthalene acetic acid	86-87-3	solid	NC	77.96	61.14	78.04	72.51	78.78	65.63	94.52	98.46	109.52
14	Allyl phenoxy-acetate	7493-74-5	liquid	NC	92.50	71.28	71.67	74.87	89.00	88.65	68.96	61.69	81.93
15	Isopropanol	67-63-0	liquid	NC	67.94	65.27	77.09	99.49	78.23	70.66	81.49	74.11	75.17
16	4-Methyl-thio-benzaldehyde	3446-89-7	liquid	NC	-0.85	0.08	5.33	12.96	9.72	5.26	3.58	10.69	27.42
17	Methyl stearate	112-61-8	solid	NC	91.44	87.87	101.09	84.86	72.50	87.20	102.60	101.17	112.19
18	Heptyl butyrate	5870-93-9	liquid	NC	93.13	78.52	89.85	92.56	70.89	70.94	84.40	95.87	109.76
19	Hexyl salicylate	6259-76-3	liquid	NC	107.89	81.31	68.88	90.57	79.73	89.25	88.69	92.08	96.23
20	Cinnamaldehyde	104-55-2	liquid	NC	4.85	2.61	-1.93	1.65	1.80	1.28	-1.16	2.15	14.53

Lab 1: Biosolution Co., Lab 2: Korea Conformity Laboratories, Lab 3: COSMAX
Red cells: determined as irritants

The predictive capacity, based on 50% cutoff, of the respective laboratory analyses for a set of the 20 reference chemicals obtained during reproducibility tests were similar, with the sensitivity ranging from 96.7% to 100%, and the specificity 70% for all laboratories, and the accuracy ranging from 83.3% to 85% (Table 13).

[Table 13] Predictive capacity estimated on individual run base or substance base for the 20 reference chemicals

		Individual run base				Substance base			
	PS	Lab 1	Lab 2	Lab 3	Total	Lab 1	Lab 2	Lab 3	Average
Sensitivity	≥ 80%	**100% (30/30)**	**100% (30/30)**	**96.7% (29/30)**	**98.9% (89/90)**	**100% (10/10)**	**100% (10/10)**	**100% (10/10)**	**100%**

Specificity	≥ 70%	**70.0% (21/30)**	**70.0% (21/30)**	**70.0% (21/30)**	**70.0% (63/90)**	**70.0% (7/10)**	**70.0% (7/10)**	**70.0% (7/10)**	**70.0%**
Accuracy	≥ 75%	**85.0 % (51/60)**	**85.0% (51/60)**	**83.3% (50/60)**	**84.4% (152/180)**	**85.0 % (17/20)**	**85.0 % (17/20)**	**85.0 % (17/20)**	**85.0%**

Lab 1: Biosolution Co., Lab 2: Korea Conformity Laboratories, Lab 3: COSMAX

According to both individual run-based approach (all 60 runs [3 runs x 20 chemicals] in each lab were considered, producing 60 predictions for each lab) and substance-based approach (prediction made with the average cell viability of 3 runs for each chemical in each lab was considered, resulting in 20 predictions for each lab), the results indicated all three values of predictive capacity satisfactorily met the acceptance criteria given in the OECD GD 220.

6.2 Colour Interference

All three participating labs conducted the correction procedure for colour interference as described in VII 1.2 for the 20 reference chemicals to check the possibility of direct staining and/or MTT reactivity. In Biosolution Co., it was concluded that the test results were not needed to be corrected for colour interferences since no direct staining was shown and the 7 chemicals exhibiting reactivity were all determined to be irritants even without correcting colour interference. The results were as follows.

[Table 14] Colour interference check for the 20 PS reference chemicals in Biosolution Co.

No.	Chemical	Uncorrected cell viability (BS)			Decision	Direct Staining	MTT reactivity	Final CV (%)	Correction
		2019.01	2019.02	2019.03					
1	1-Decanol	8.94	2.28	11.26	I				
2	Cyclamen aldehyde	5.69	3.8	13.11	I		O	2.14 , 0.10, 5.01	X
3	1-Bromohexane	18.58	4.77	7.1	I				
4	2-Chloromethyl-3,5-dimethyl-4-methoxypyridine HCl	8.03	5.46	6.74	I				
5	Di-n-propyl disulphide	24.6	24.69	27.5	I				
6	Potassium hydroxide(5% aq.)	16.1	3.2	8.73	I		O	1.80, -2.50, -1.17	X
7	Benzenethiol, 5-(1,1-dimethylethyl)-2-methyl	31.73	20.43	40.26	I		O	7.33, 6.53, 5.66	X
8	1-Methyl-3-phenyl-1-piperazine	4.95	7.11	11.16	I		O	-0.95, 1.21, 4.16	X
9	Heptanal	14.96	6.21	16.46	I		O	8.41, 4.36, 2.61	X
10	Tetrachloroethylene	9.45	2.94	7.56	I				
11	1-Bromo-4-chlorobutane	15.85	20.97	20.35	I				
12	Diethyl phthalate	82.13	74.96	88.58					
13	Naphthalene acetic acid	77.96	61.14	78.04					
14	Allyl phenoxy-acetate	92.5	71.28	71.67					
15	Isopropanol	67.94	65.27	77.09					
16	4-Methyl-thio-benzaldehyde	11.7	4.23	12.48	I		O	-0.85, 0.08, 5.33	X
17	Methyl stearate	91.44	87.87	101.09					
18	Heptyl butyrate	93.13	78.52	89.85					

19	Hexyl salicylate	107.89	81.31	68.88					
20	Cinnamaldehyde	14.3	6.96	10.92	I		O	4.85, 2.61, -1.93	X

Red cell is irritant based on uncorrected cell viability; O: MTT reactivity, X: correction unnecessary

KCL also conducted the colour intereference examination through MTT assay and the results concluded that the colour intereference did not affect the prediction decision made with uncorrected viability, and the results are shown as below.

[Table 15] Colour Interference check for the 20 PS reference chemicals in KCL

No.	Chemical	Uncorrected cell viability (KCL)			Decision	Direct Staining	MTT reactivity	Final CV (%)	Correction
		2019.01	2019.02	2019.03					
1	1-Decanol	8.56	3.21	10.18	I				
2	Cyclamen aldehyde	7.61	1.6	5.42	I		O	4.01, 1.10, 2.62	X
3	1-Bromohexane	10.11	8.23	13.07	I				
4	2-Chloromethyl-3,5-Dimethyl-4-methoxypyridine HCl	1.91	1.85	2.47	I				
5	Di-n-propyl disulphide	4.5	10.72	14.39	I		O	2.45, 8.22, 14.39	X
6	Potassium hydroxide(5% aq.)	2.13	2.05	1	I		O	0.43, -0.25, -0.95	X
7	Benzenethiol, 5-(1,1-dimethylethyl)-2-methyl	8.09	8.68	12.37	I		O	-5.86, -11.12, 0.62	X
8	1-Methyl-3-phenyl-1-Piperazine	1.57	5.21	4.3	I		O	0.27, 4.46, 1.55	X
9	Heptanal	3.37	2.55	5.8	I		O	1.32, -0.85, 2.70	X
10	Tetrachloroethylene	7.1	6.09	6.66	I				
11	1-Bromo-4-chlorobutane	5.92	2.9	9.31	I				
12	Diethyl phthalate	94.33	91.62	82.17					
13	Naphthalene acetic acid	72.51	78.78	65.63					
14	Allyl phenoxy-acetate	74.87	89	88.65					
15	Isopropanol	99.49	78.23	70.66					
16	4-Methyl-thio-benzaldehyde	16.56	11.97	9.46	I		O	12.96, 9.72, 5.26	X
17	Methyl stearate	84.86	72.5	87.2					
18	Heptyl butyrate	92.56	70.89	70.94					
19	Hexyl salicylate	90.57	79.73	89.25					
20	Cinnamaldehyde	7.5	7	7.53	I		O	1.65, 1.80, 1.28	X

Red cell is irritant based on uncorrected cell viability; O: MTT reactivity, X: correction unnecessary

COSMAX also showed similar results in the colour interference examination with the other two laboratories except for Benzenethiol, 5-(1,1-dimethylethyl)-2-methyl (No.7), which was determined as non-irritant with the uncorrected cell viability. However, the prediction decision was changed to 'irritant' via colour interference correction. The results are shown in Table 16.

[Table 16] Colour Interference check for the 20 PS reference chemicals in COSMAX

No.	Chemical	Uncorrected cell viability (COSMAX)			Decision	Direct Staining	MTT reactivity	Final CV (%)	Correction
		2019.03	2019.04	2019.05					
1	1-Decanol	12.18	13.48	13.69	I				

2	Cyclamen aldehyde	6.71	7.65	5.83	I		O	2.41, -5.85, 3.68	X
3	1-Bromohexane	41.57	33.80	33.18	I				
4	2-Chloromethyl-3,5-dimethyl-4-methoxypyridine HCl	2.20	2.04	2.94	I				
5	Di-n-propyl disulphide	38.18	38.22	62.51	I				
6	Potassium hydroxide(5% aq.)	5.90	1.32	3.01	I		O	-3.85, -4.48, 0.36	X
7	Benzenethiol, 5-(1,1-dimethylethyl)-2-methyl	44.78	53.69	60.08	NI/I		O	-1.17,-4.46, 20.93	O*
8	1-Methyl-3-phenyl-1-piperazine	16.15	30.84	3.39	I		O	9.35, 30.84, -1.86	X
9	Heptanal	7.31	4.21	6.87	I		O	-1.29, 4.21, 3.72	X
10	Tetrachloroethylene	39.74	12.59	34.70	I				
11	1-Bromo-4-chlorobutane	3.38	4.54	23.57	I				
12	Diethyl phthalate	74.06	101.78	103.28					
13	Naphthalene acetic acid	94.52	98.46	109.52					
14	Allyl phenoxy-acetate	68.96	61.69	81.93					
15	Isopropanol	81.49	74.11	75.17					
16	4-Methyl-thio-benzaldehyde	8.98	15.24	29.72	I		O	3.58, 10.69, 27.42	X
17	Methyl stearate	102.60	101.17	112.19					
18	Heptyl butyrate	84.40	95.87	109.76					
19	Hexyl salicylate	88.69	92.08	96.23					
20	Cinnamaldehyde	10.79	11.40	14.53	I		O	-1.16, 2.15, 14.53	X

Red cell is irritant based on uncorrected cell viability: O: MTT reactivity, X: correction unnecessary
*: correction necessary

Details on the colour interference tests were provided in Appendix 12.

IX. Discussion

1. In Consideration of Invalid Runs

In accordance with the re-test criteria #3 and #4, namely the standard deviation (SD) between three tissues replicates (negative control, positive control, and test chemicals) exceed 18%, and the viability fall between 45% and 55%, there were two cases of the re-tests. Of the total 180 determinations, re-test was carried out for two substances (1 for criteria #3 and 1 for criteria #4). The re-test was done by COSMAX and there was no re-test by Biosolution Co. and KCL. However, all invalid results obtained from COSMAX were resolved after the first round of re-test. As a result, complete runs of three repetitions were accomplished for the 20 PS chemicals by all three laboratories.

2. Reliability

WLRs were 100% at Lab 1 and 2, and 95% at Lab 3, meeting the requirements specified in the TG 439 performance standards, which is to be ≥ 90%. BLR for all three participating laboratories was

100%, meeting the ≥ 80% criteria as specified in the TG 439 performance standards. The results demonstrate that the robustness and reliability of the test method are excellent to meet the performance standards in OECD GD 220.

3. Predictive Capacity

All three laboratories completed three valid runs successfully. Almost all UN GHS Category 2 chemicals were correctly determined as irritants except for one false negative determination of Di-n-propyl disulphide (No. 5, Category 2) by Lab 3.

Sensitivity was determined in the range of 96.7% to 100% at each laboratory, and when a total of 180 individual runs were considered as a whole, the result showed 98.9% of the sensitivity, which satisfactorily met the sensitivity criterion stated in the OECD GD 220 performance standards, ≥ 80% (Table 13).

In the case of false positives (Table 17), there were 3 false positive determinations: 1-bromo-4-chlorobutane (No. 11), 4-methyl-thio-benzaldehyde (No. 16), and cinnamaldehyde (No. 20) were false positives in all runs by all three laboratories. Chemicals No. 11, 16 and 20 were also shown as false positives in the Epiderm™ SIT.

Specificity was calculated to be 70% for all three laboratories, and taking into account all individual runs, 70% of specificity was obtained from a total of 180 runs, which met the specificity criteria of ≥ 70% as specified in the OECD GD 220 performance standards.

Likewise, the accuracy was determined ranging from 83.3% to 85% at each laboratory, and when a total of 180 individual runs were considered, the results showed 84.4% accuracy, meeting an accuracy criterion of ≥ 75% as stated in the OECD GD 220 performance standards (Table 13).

[Table 17] Overview of misclassified chemicals in the PS

PS No.	Chemical	CAS No.	UN GHS	Lab 1			Lab 2			Lab 3		
				1	2	3	1	2	3	1	2	3
5	Di-n-propyl disulphide	629-19-6	Cat. 2	TP	TP	TP	TP	TP	TP	TP	TP	FN
11	1-Bromo-4-chlorobutane	6940-78-9	NC	FP	FP	FP	FP	FP	FP	FP	FP	FP
16	4-Methyl-thio-benzaldehyde	3446-89-7	NC	FP	FP	FP	FP	FP	FP	FP	FP	FP
20	Cinnamaldehyde	104-55-2	NC	FP	FP	FP	FP	FP	FP	FP	FP	FP

TP: True positive, TN; True negative, FP; False positive, FN; False negative
Lab 1: Biosolution Co., Lab 2; Korea Conformity Laboratories, Lab 3; COSMAX

3.1 Predictive Capacity of KeraSkin™ SIT for 66 Chemicals in Total

To further assess the predictive capacity of the KeraSkin™ SIT, additional 46 test chemicals with known *in vivo* irritancy supported by high quality *in vivo* evidence, composed of 27 liquids and 19 solids (4 corrosives, 7 irritants and 35 non-irritants) were selected from the previous validation study reports and scientific papers. The composition of the 46 test chemicals is as below (Table 18) and the details on their identity and source references can be found in Appendix 2 and Annex 1.

[Table 18] Composition of additional 46 chemicals used in the evaluation of the predictive capacity

UN GHS	VRM	Total	Liquid (27)	Solid (19)
Category 1 (Corrosive)	**Irritant**	4	3	1
Category 2 (Irritant)		7	6	1
Category 3	**Non-irritant**	2	2	0
No Category		33	16	17

The data were produced in compliance with protocol 1.5 by Biosolution Co. and KCL in a coded fashion. The test results are summarized in Table 19, which were corrected for colour interference.

[Table 19] List of 46 chemicals tested for predictive capacity and KeraSkin™ SIT CVs (%)

No	Chemical	CAS No.	Physical state	UN GHS	In vivo class	CV (%)
1	Nonanoic acid	112-05-0	liquid	Cat 2	I	2.99
2	Butyl methacrylate	97-88-1	liquid	Cat 2	I	24.64
3	Butyric acid	107-92-6	liquid	Cat 1B	I	2.19
4	Heptanoic acid	111-14-8	liquid	Cat 1B	I	2.17
5	Decanoic acid	334-48-5	liquid	Cat 2	I	43.59
6	1-Bromopentane	110-53-2	liquid	Cat 2	I	11.56
7	alpha-Terpineol	98-55-5	liquid	Cat 2	I	17.09
8	Octanoic acid (Caprylic acid)	124-07-2	liquid	Cat 1B/1C	I	1.96
9	Polyethylene glycol 400	25322-68-3	liquid	NC	NI	96.08
10	(3-Chloropropyl) trimethoxysilane	2530-87-2	liquid	NC	NI	3.34
11	3,3-Dithiodipropionic acid	1119-62-6	solid	NC	NI	96.31
12	2-Phenylethanol	60-12-8	liquid	NC	NI	0.29
13	Benzyl salicylate	118-58-1	liquid	NC	NI	98.45
14	1-(4-Chlorophenyl)-3-(3,4-dichlorophenyl)urea	101-20-2	solid	-	NI	102.27
15	3,3′-Dithiodipropionic acid	562-49-2	liquid	NC	NI	88.30
16	4.4-Methylenebis (2,6-di-tert-butylphenol)	118-82-1	solid	NC	NI	97.28
17	Dodecanoic acid	143-07-7	solid	NC	NI	84.81
18	1-Chloro-3-nitrobenzene	121-73-3	solid	NC	NI	87.72
19	Benzyl benzoate	120-51-4	liquid	NC	NI	98.34
20	2-(Formylamino)-3-thiophenecarboxylic acid	43028-69-9	solid	NC	NI	100.94
21	Sodium bisulphite	7631-90-5	solid	NC	NI	9.39
22	4-Acetoxy-2,5-dimethyl-3(2H)furanone	4166-20-5	liquid	NC	NI	4.58
23	4-Amino-4H-1,2,4-triazole	584-13-4	solid	NC	NI	89.65
24	Di-propylene glycol	25265-71-8	liquid	NC	NI	94.86
25	Di(propylene glycol) butyl ether, mixture of isomers	29911-28-2	liquid	NC	NI	13.86
26	Erucamide	112-84-5	solid	NC	NI	103.10
27	Propylene glycol	57-55-6	liquid	NC	NI	84.53
28	Triethylene glycol	112-27-6	liquid	NC	NI	76.62
29	Sodium bicarbonate	144-55-8	solid	NC	NI	90.95
30	Isopropyl palmitate	142-91-6	liquid	NC	NI	105.99
31	Isopropyl myristate	110-27-0	liquid	NC	NI	101.94
32	2-Ethylhexyl 4-methoxycinnamate	5466-77-3	liquid	NC	NI	106.45

33	Tetrabromophenol blue	4430-25-5	solid	NC	NI	89.89
34	Piroctone olamine	68890-66-4	solid	Cat 2	I	1.71
35	25% Cetyltrimethylammonium chloride solution (in D.W.)	112-02-7	liquid	Cat 2	I	1.07
36	Ferric chloride	7705-08-0	solid	Cat 1B	I	57.74
37	2-Hydroxy-4-methoxybenzophenone, Oxybenzone	131-57-7	solid	NC	NI	99.25
38	Cyclohexadecanone	2550-52-9	solid	NC	NI	103.94
39	Methyl laurate	111-82-0	liquid	NC (Cat 3)	NI	91.17
40	Capryl-isostearate	209802-43-7	liquid	NC	NI	94.08
41	2-Ethylhexyl-4-aminobenzoate	26218-04-2	solid	NC	NI	103.13
42	2-Phenylhexanenitrile	3508-98-3	liquid	NC (Cat 3)	NI	76.00
43	Barium sulfate	7727-43-7	solid	-	NI	94.83
44	Diisopropyl sebacate	2042106	liquid	-	NI	93.84
45	10% Xanthan gum (in D.W.)	11138-66-2	gel	-	NI	99.63
46	3-Chloro-4-fluoronitrobenzene	350-30-1	solid	NC	NI	80.67

CV: Cell viability (%), Red colour, false determinations.

The data for 66 test chemicals in total (46 chemicals tested to evaluate predictive capacity of KeraSkin™ SIT and the 20 reference chemicals in OECD GD220) with known *in vivo* irritancy, composed of 43 liquids and 23 solids (1 gel chemical was regarded as solid), or 21 irritants (4 corrosives, 17 irritants) and 45 non-irritants were evaluated to assess the predictive capacity of KeraSkin™ SIT. The results as shown in Table 20, indicated that KeraSkin™ SIT has performance that meets the PS criteria.

[Table 20] Assessment of the predictive capacity with 66 chemicals in total

	Total (66)		**Liquid (43)**		**Solid (23)**		**PS criteria**
	I	**NI**	**I**	**NI**	**I**	**NI**	
I	20	1	17	0	3	1	
NI	8	37	7	19	1	18	
total	66		43		23		
Sensitivity	**95.2%**		100%		75%		≥ 80%
Specificity	**82.2%**		73.1%		94.7%		≥ 70%
Accuracy	**86.4%**		83.7%		91.3%		≥ 75%

There was one false negative determination and 5 false positive determinations among 46 additional chemicals. (3-Chloropropyl)trimethoxysilane, 2-Phenylethanol, Sodium bisulphite, 4-Acetoxy-2,5-dimethyl-3(2H)furanone, and Di(propylene glycol) butyl ether, mixture of isomers were false positives. False positive results for these chemicals were also frequently observed with other existing VRMs. Ferric chloride (III) was a false negative. However, Ferric chloride (III) was determined a true positive in an earlier study (Kandarova et al., 2009). Since the result of false negative is considered to be from the instability of the chemical, re-tests were conducted with newly opened chemical and different MTT concentrations according to peer reviewer's comment. However, the results were similar, indicating that this is a false negative for KeraSkin™ SIT. Further details on additional test can be found

in the Annex 1 of this validation report.

3.2 Weighted Approach in Assessing the Predictive Capacity of KeraSkin™ SIT for 66 Chemicals

The predictive capacity of KeraSkin™ SIT can be re-assessed with a weighted approach for 66 test chemicals since some chemicals have been tested more than other chemicals, i.e. the number of tests was not the same across chemicals.

[Table 21] Comparison of the run-based estimation of predictive capacity of KeraSkin™ SIT using a weighted approach for 66 chemicals

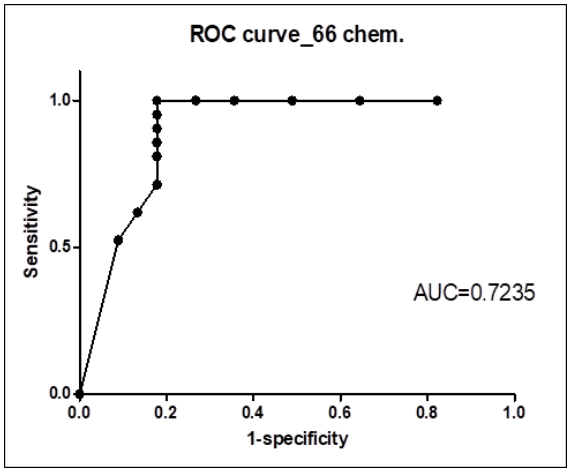
Predictive capacity	PS criteria	Weighted			Unweighted*		
		Total (66)	Liquid (43)	Solid (23)	Total (66)	Liquid (43)	Solid (23)
Sensitivity	≥ 80%	**94.7%**	99.3%	75%	**95.2%**	100%	75%
Specificity	≥ 70%	**82.2%**	73.1%	94.7%	**82.2%**	73.1%	94.7%
Accuracy	≥ 75%	**86.2%**	83.5%	91.3%	**86.4%**	83.7%	91.3%

*Run-based approach without weighted analysis

As shown in Table 21, the predictive capacity of KeraSkin™ SIT met the criteria of PS regardless of weighted analysis.

3.3 Estimation of the Optimal Cutoff for Keraskin™ SIT with 66 Chemicals

The optimal cutoff to maximize the predictive capacity of the Keraskin™ SIT was estimated with the viability data for 66 test chemicals through ROC (receiver operating characteristic) curve analysis as below in Figure 9. ROC curve represents general performance of assay or diagnostics. Based on the statistical analysis, the cutoff value of 60-75% was determined to be optimal for the maximization of sensitivity as well as accuracy (Table 22: sensitivity of 100%, specificity of 82.2% and accuracy of 78.4%). However, Keraskin™ SIT showed a reasonable predictive capacity with the 50% cutoff for 66 test chemicals with the AUC of 0.7235, which represents a reasonable level of predictive capacity.



[Figure 9] ROC curve of the predictive capacity for 66 chemicals

[Table 22] Predictive capacity for 66 chemicals with varying cutoffs

Cutoff	Sensitivity	Specificity	Accuracy	1-Specificity
5	0.524	0.911	0.703	0.089
10	0.619	0.867	0.703	0.133
15	0.714	0.822	0.703	0.178
20	0.810	0.822	0.730	0.178
25	0.857	0.822	0.743	0.178
30	0.905	0.822	0.757	0.178
35	0.905	0.822	0.757	0.178
40	0.905	0.822	0.757	0.178
45	0.952	0.822	0.770	0.178
50	0.952	0.822	0.770	0.178
55	0.952	0.822	0.770	0.178
60	1.000	0.822	0.784	0.178
65	1.000	0.822	0.784	0.178
70	1.000	0.822	0.784	0.178
75	1.000	0.822	0.784	0.178
80	1.000	0.733	0.730	0.267
85	1.000	0.644	0.676	0.356
90	1.000	0.511	0.595	0.489
95	1.000	0.356	0.500	0.644
100	1.000	0.178	0.392	0.822

yellow shaded indicates best predictive capacity.

3.4 Predictive Capacity of KeraSkin™ SIT for 62 Chemicals After Removing 4 Corrosive Chemicals

There were 4 corrosive chemicals (Butyric acid, Heptanoic acid, Octanoic acid and Ferric chloride) included in 66 chemicals. Since OECD TG 439 aims to identify irritant chemicals from non-irritant chemicals, those 4 corrosive chemicals may be misleading the predictive capacity of KeraSkin™ SIT. Therefore, after removing those corrosive chemicals, predictive capacity was re-analyzed with 62 chemicals as shown below.

[Table 23] Assessment of the predictive capacity with 62 chemicals without 4 corrosive chemicals

	Total (62)		Liquid (40)		Solid (22)		PS criteria
	I	NI	I	NI	I	NI	
I	17	0	14	0	3	0	
NI	8	37	7	19	1	18	
total	62		40		22		
Sensitivity	**100%**		100%		100%		≥ 80%
Specificity	**82.2%**		73.1%		94.7%		≥ 70%
Accuracy	**87.1%**		82.5%		95.5%		≥ 75%

As shown above, KeraSkin™ SIT shows 100% sensitivity for liquids and solids while specificity is higher for solids (94.7% vs 73.1%).

3.5 Weighted Approach in Assessing the Predictive Capacity of KeraSkin™ SIT for 62 Chemicals

The predictive capacity of KeraSkin™ SIT can be re-assessed with a weighted approach for 62 test chemicals.

[Table 24] Comparison of the run-based estimation of predictive capacity of KeraSkin™ SIT using a weighted approach for 62 chemicals

Predictive capacity	PS criteria	Weighted			Unweighted*		
		Total (62)	Liquid (40)	Solid (22)	Total (62)	Liquid (40)	Solid (22)
Sensitivity	≥ 80%	**99.3%**	99.2%	100%	**100%**	100%	100%
Specificity	≥ 70%	**82.2%**	73.1%	94.7%	**82.2%**	73.1%	94.7%
Accuracy	≥ 75%	**86.9%**	82.2%	95.5%	**87.1%**	82.5%	95.5%

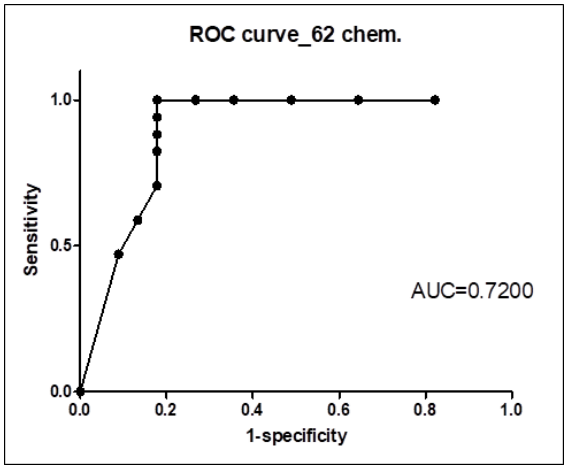
*Run-based approach without weighted analysis

As shown in Table 24, the predictive capacity of KeraSkin™ SIT met the criteria of PS regardless of weighted analysis.

3.6 Estimation of the Optimal Cutoff for KeraSkin™ SIT with 62 Chemicals

The optimal cutoff to maximize the predictive capacity of the KeraSkin™ SIT was re-estimated with the viability data for 62 test chemicals through ROC (receiver operating characteristic) curve analysis as below in Figure 10. Based on the statistical analysis, the cutoff value of 45-75% was determined to be optimal for the maximization of sensitivity as well as accuracy for 62 chemicals (Table 25: sensitivity of 100%, specificity of 82.2% and accuracy of 73%). Re-analysis of KeraSkin™ SIT for

62 test chemicals showed the AUC of 0.7200.



[Figure 10] ROC curve of the predictive capacity for 62 chemicals

[Table 25] Predictive capacity for 62 chemicals with varying cutoffs

Cutoff	Sensitivity	Specificity	Accuracy	1-Specificity
5	0.471	0.911	0.662	0.089
10	0.588	0.867	0.662	0.133
15	0.706	0.822	0.662	0.178
20	0.824	0.822	0.689	0.178
25	0.882	0.822	0.703	0.178
30	0.941	0.822	0.716	0.178
35	0.941	0.822	0.716	0.178
40	0.941	0.822	0.716	0.178
45	1.000	0.822	0.730	0.178
50	1.000	0.822	0.730	0.178
55	1.000	0.822	0.730	0.178
60	1.000	0.822	0.730	0.178
65	1.000	0.822	0.730	0.178
70	1.000	0.822	0.730	0.178
75	1.000	0.822	0.730	0.178
80	1.000	0.733	0.676	0.267
85	1.000	0.644	0.622	0.356
90	1.000	0.511	0.541	0.489
95	1.000	0.356	0.446	0.644

100	1.000	0.178	0.338	0.822

3.7 Colour Interference

Colour interference is a potential issue for MTT assay-based in vitro tests. While Biosolution Co. (Lab. 1) and KCL (Lab. 2) ensured that no correction was necessary for the 20 PS substances, COSMAX (Lab. 3) found that prediction decision was changed for one chemical, benzenethiol, 5-(1,1-dimethylethyl)-2-methyl (No.7), which was determined as non-irritant with the uncorrected cell viability but the prediction decision was changed to 'irritant' via colour interference correction. This suggests that the KeraSkin™ SIT needs colour interference test similar as other VRMs of OECD TG439.

Among 46 chemicals tested for additional predictive capacity assessment, 10 chemicals showed concern of colour interference (all were MTT reducing chemicals), but correction did not change the prediction decision as shown below. Details of the colour interference study were included in Appendix 12.

[Table 26] Overview of 10 colour interfering chemicals among additional 46 chemicals

No.	Chemical	CAS No.	Physical state	UN GHS	in vivo class	Uncorrected cell viability		Decision	Direct Staining	MTT reactivity	Both	Final CV (%)	Decision change
						BS	KCL						
15	Silane A-1430	2530-87-2	liquid	NC	NI	9.8		I		6.5		3.3	X
17	Phenylethylalcohol	60-12-8	liquid	NC	NI	3.2		I		2.9		0.3	X
26	Sodium bisulphite	7631-90-5	solid	NC	NI		10.8	I		1.5		9.4	X
27	2,5-Dimethyl-4-oxo-4,5-dihydrofuran-3yl acetate	4166-20-5	liquid	NC	NI	7.6		I		3.1		4.6	X
38	Tetrabromophenol blue	4430-25-5	solid	NC	NI		99.5	NI		5.5		94	X
41	Piroctone olamine	68890-66-4	solid	Cat 2	I	4.8		I		3.1		1.7	X
42	25% Cetrimonium chloride (in D.W.)	112-02-7	liquid	Cat 2	I		4.3	I		3.3		1.1	X
50	2-Phenylhexanenitrile	3508-98-3	liquid	NC	NI	80.1		NI		4.1		76.0	X
51	Barium sulfate	7727-43-7	solid	-	NI		97.1	NI		2.3		94.8	X
52	Diisopropyl sebacate	2042106	liquid	-	NI		95.7	NI		1.9		93.8	X

4. Similarity with the OECD TG 439 VRM

The VMT considers the KeraSkin™ SIT to be structurally and functionally similar to RhE SIT VRMs in OECD TG 439. Likewise, there are procedural similarities in each of essential test component (Table 3). With the 20 reference chemicals from the performance standards, the KeraSkin™ SIT resulted in a predictive capacity comparable to the VRMs.

5. Limitation of the KeraSkin™ SIT

The VMT considers that, as similar with other SITs utilizing RhE, a limitation of the KeraSkin™ SIT is that it does not allow discrimination between skin irritation/reversible effects (Category 2) and

skin corrosion/irreversible damage (Category 1), nor it does allow the classification of chemicals to the optional UN GHS Category 3 (mild irritants). For these purposes, further testing with other *in vitro* test methods as described in IATA is required.

X. Conclusion

This validation study aimed to investigate if the KeraSkin™ SIT is capable of fulfilling the performance standards stipulated in OECD GD 220 for similar or modified *in vitro* RhE SIT methods. The assessment of reliability and relevance of the KeraSkin™ SIT method was performed using the 20 reference chemicals listed in the performance standards, OECD GD 220. Having achieved a WLR of 95% to 100% in all three participating laboratories and a BLR of 100% in the three participating laboratories, the KeraSkin™ SIT was considered to satisfy the criteria of performance standards, OECD GD 220.

The KeraSkin™ SIT demonstrated a predictive capacity with an overall accuracy of 84.4%, a sensitivity of 98.9% and a specificity of 70.0% for the 20 reference chemicals, when all 180 runs were considered. In additional predictive capacity analysis, a sensitivity of 95.2%, a specificity of 82.2% and an accuracy of 86.4% were obtained for 66 chemicals and a sensitivity of 100%, a specificity of 82.2% and an accuracy of 87.1% were obtained for 62 chemicals after removing corrosive chemicals, meeting the criteria of 75% for accuracy, 80% for sensitivity, and 70% for specificity stipulated in the OECD GD 220 performance standards. These results suggest that the KeraSkin™ SIT may be included as an additional validated PS based test method in OECD TG 439 for predicting the potential of skin irritation.

XI. Acknowledgements

The VMT would like to thank the MFDS (Ministry of Food and Drug Safety, Korea) for its generosity in supporting this validation study with a grant (18182MFDS463).

XII. References

Akbarsha, M. A., & Mascarenhas, B. (2019). Cosmetic Regulation and Alternatives to Animal Experimentation in India *Alternatives to Animal Testing* (pp. 57-62): Springer.

Choi, J., Kim, H., Choi, J., Oh, S. M., Park, J., & Park, K. (2014). Skin corrosion and irritation test of sunscreen nanoparticles using reconstructed 3D human skin model. *Environ Health Toxicol, 29*, e2014004. doi:10.5620/eht.2014.29.e2014004

Hwang, J. H., Park, H., Choi, D. W., Nam, K. T., & Lim, K. M. (2018). Investigation of dermal toxicity of ionic liquids in monolayer-cultured skin cells and 3D reconstructed human skin models. *Toxicol In Vitro, 46*, 194-202. doi:10.1016/j.tiv.2017.09.025

Jung, K. M., Lee, S. H., Jang, W. H., Jung, H. S., Heo, Y., Park, Y. H., . . . Seok, S. H. (2014). KeraSkin-VM: a novel reconstructed human epidermis model for skin irritation tests. *Toxicol In Vitro, 28*(5), 742-750. doi:10.1016/j.tiv.2014.02.014

Kim, K., Leutou, A. S., Jeong, H., Kim, D., Seong, C. N., Nam, S. J., & Lim, K. M. (2017). Anti-Pigmentary Effect of (-)-4-Hydroxysattabacin from the Marine-Derived Bacterium Bacillus sp. *Mar Drugs, 15*(5). doi:10.3390/md15050138

MatTeK (2002) Skin Irritation (MTT ET-50) *https://www.mattek.com/application/skin-irritation-mtt-et-50/* accessed on 13th Jan 2021

OECD. (2005). *Test No. 34: Guidance Document on the Validation and International. Acceptance of New or Updated Test Methods for Hazard. Assessment* Paris: OECD Publishing Retrieved from http://www.oecd.org/env/testguidelines.

OECD. (2015a). *No. 220 Performance Standards for the Assessment of Proposed Similar or Modified in vitro Reconsructed Human Epidermis (RhE) Test Methods for Skin Irritation Testing as described in TG 439*. Paris: OECD Publishing Retrieved from http://www.oecd.org/env/ehs/testing/performancestandardsfortheassessmentofproposedsimilarormodifiedinvitroreconstructedhumancornea-likeepitheliumrhcetestmethodsforeyehazard.htm.

OECD. (2015b). *Test No. 404: Acute Dermal Irritation/Corrosion*.

OECD. (2015c). *Test No. 439: In Vitro Skin Irritation - Reconstructed Human Epidermis Test Method*: OECD Publishing.

OECD. (2017). *Guidance Document on an Integrated Approach on Testing and Assessment (IATA) for Skin Corrosion and Irritation*. Paris: OECD Publishing Retrieved from https://www.oecd.org/publications/guidance-document-on-an-integrated-approach-on-testing-and-assessment-iata-for-skin-corrosion-and-irritation-9789264274693-en.htm.

OECD. (2019). *Test No. 439: In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method*: OECD Publishing.

Annex 1

Peer Review Panel comments and Answers from KeraSkin™ SIT validation management team (VMT)

1. Information about test method development and optimization

Q: Please provide the list of chemicals used to optimise the protocol of KeraSkin™ SIT method.

A: The list of chemicals used during protocol development and optimization for each Protocol version. We added the list in the revised validation report (Table 6).

Table 1. List of chemicals used for protocol development and optimization

Version (approval date)	Revision	Chemicals used	Rationale
Protocol 1.0 (Apr 15th 2012)		**20% SLS**	
Protocol 1.1 (Apr 8th 2013)	**Addition of the MTT and Nylon Mesh reactivity assay for test chemicals**	**20% SLS**	
Protocol 1.2 (Sep 7th 2015)	**Changes in the washing procedure**	**1,5-hexadiene (CAS RN 592-42-7)** **3,3-Dimethylpentane (CAS RN 562-49-2)** **Isopropanol (CAS RN 67-63-0)**	**Chemicals with issues in washing**
Protocol 1.3 (Dec 1st 2016)	**Addition of frozen tissue production method and interference test flow chart** **Changes in the washing procedure**	**1-bromobutane (CAS RN 109-65-9)** **1-bromopentane (CAS RN 110-53-2)** **1-bromohexane (CAS RN 111-25-1)** **Allyl phenoxy acetate (CAS RN 7493-74-5)** **Heptyl butyrate (CAS RN 5870-93-9)**	**- Persisting chemicals**
Protocol 1.4 (Apr 23rd 2018)	**Changes in the QC criteria in the laboratory** **Change MTT concentration: 0.3 ㎎/㎖ → 0.4 ㎎/㎖** **Changes in the application procedure of test** **Addition of re-test criteria**	**α-terpineol (CAS RN 98-55-5)** **1-BromoPentane (CAS RN 110-53-2)** **butyl methacrylate (CAS RN 97-88-1)** **Capric acid (decanoic acid) (CAS RN 334-48-5)** **Allyl phenoxy acetate (CAS RN 7493-74-5)** **1-bromohexane (CAS RN 111-25-1)** **Di-n-propyldisulphide (CAS RN 629-19-6)** **3,3-Dithiodipropionic Acid (CAS RN 1119-62-6)** **4-Amino-1,2,4-Triazole (CAS RN 584-13-4)** **Erucamide (CAS RN 112-84-5)** **Benzyl Salicylate (CAS RN 118-58-1)** **Sodium Bisulphite (CAS RN 7631-90-5)**	**Falsely predicted chemicals**
Protocol 1.5 (Jan 11th 2019)	**Additions in washing step**	**Naphthalene acetic acid (CAS RN 86-87-3)** **3,3-Dithiodipropionic Acid (CAS RN 1119-62-6)** **4-Amino-1,2,4-Triazole (CAS RN 584-13-4)** **Erucamide (CAS RN 112-84-5)** **1,5-hexadiene (CAS RN 592-42-7)** **Allyl phenoxy acetate (CAS RN 7493-74-5)** **Polyethlene glycol 400 (CAS RN 25322-68-3)** **3,3-Dimethylpentane (CAS RN 562-49-2)** **α-terpineol (CAS RN 98-55-5)** **1-BromoPentane (CAS RN 110-53-2)** **butyl methacrylate (CAS RN 97-88-1)**	**Falsely predicted chemicals**

**** Red colour indicates PS chemicals***

When the test substance was applied according to the Protocol 1.4, the mesh used previously was removed, and the application method (the amount and the time) of test substance was altered in order to evenly distribute the substance on the surface of the artificial skin.

The amount was increased(30ul→40ul) to evenly distribute the substance and the method was optimized by shortening the treating time(45mins→30mins).

Viscous substance sometimes let the mesh stay on the skin even after the washing process, so we did not use the mesh in the test.

2. Audit of tissue production

Q: Has the KeraSkin tissue manufacturing process undergone an external audit? If so, is it possible to share the audit certificate?

A: Manufacture of KeraSkin™ has been ISO 9001-accredited, which required external audit. Certificate is as shown below, which was added to QC report page 18.

ITQA

Certificate of Registration

Bio Solution Co., Ltd.

(Seoul Techno Park, Gongneung-dong)
#519, 232, Gongneung-ro, Nowon-gu, Seoul, Korea

has been approved by ITQA Co., Ltd. to the following quality management system standards :

ISO 9001:2015

The scope of quality management system is applicable to :

1. Development and Manufacture of Cell Therapy Products
2. Development and Manufacture of Reconstructed Human Tissue Model
3. Manufacture of Cosmetic Raw Materials Using Human Stem Cell

Valid Date
From 03 May 2019 To 02 May 2022
Former Certificate : 03 May 2016
Certificate No. : 19-Q-1939-03 Date of last issue : 02 May 2019

Seong Sik Yoo

IAF JAS-ANZ

This Certificate is valid only if the organization meets the requirement continually.

ITQA Co., Ltd. 6F, 30, Pajangcheon-ro 44beon-gil, Jangan-gu, Suwon-si, Gyeonggi-do, Korea www.itqa.kr

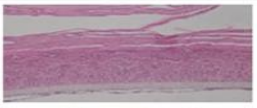
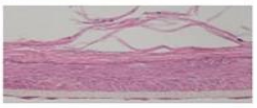
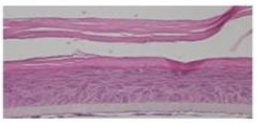
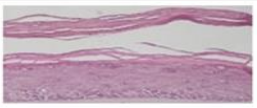
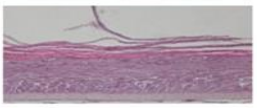
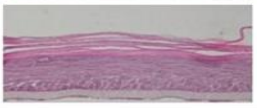
Figure 1. Certificate of registration in manufacture of KeraSkin™

3. Long-distance transportation of the model:

Q: How were the tissues transported to Shanghai (airplane, road, etc), and were the quality of the tissues (barrier function and negative controls before testing) checked in Shanghai?

A: The tissues were transported by airplane at room temperature, and the transport lasted 36-56 hours. We added the comparison of QC results between China and Korea in QC report (Table 6, page 15)

Table 2. QC results using KeraSkin™ exported to the Chinese laboratory

Laboratory	Batch No.	Tissue viability	Barrier function	Histology
		Mean OD	IC_{50} (mg/ml)	
Chinese Laboratory	KS19013	1.18	3.40	
	KS19019	1.04	2.73	
	KS19021	1.09	2.92	
Korean Laboratory	KS19013	0.88	2.78	
	KS19019	0.89	2.85	
	KS19021	0.98	3.05	

Q: Do you have data on long-distance transportation that took place during summer and/or winter, when temperatures are more extreme?

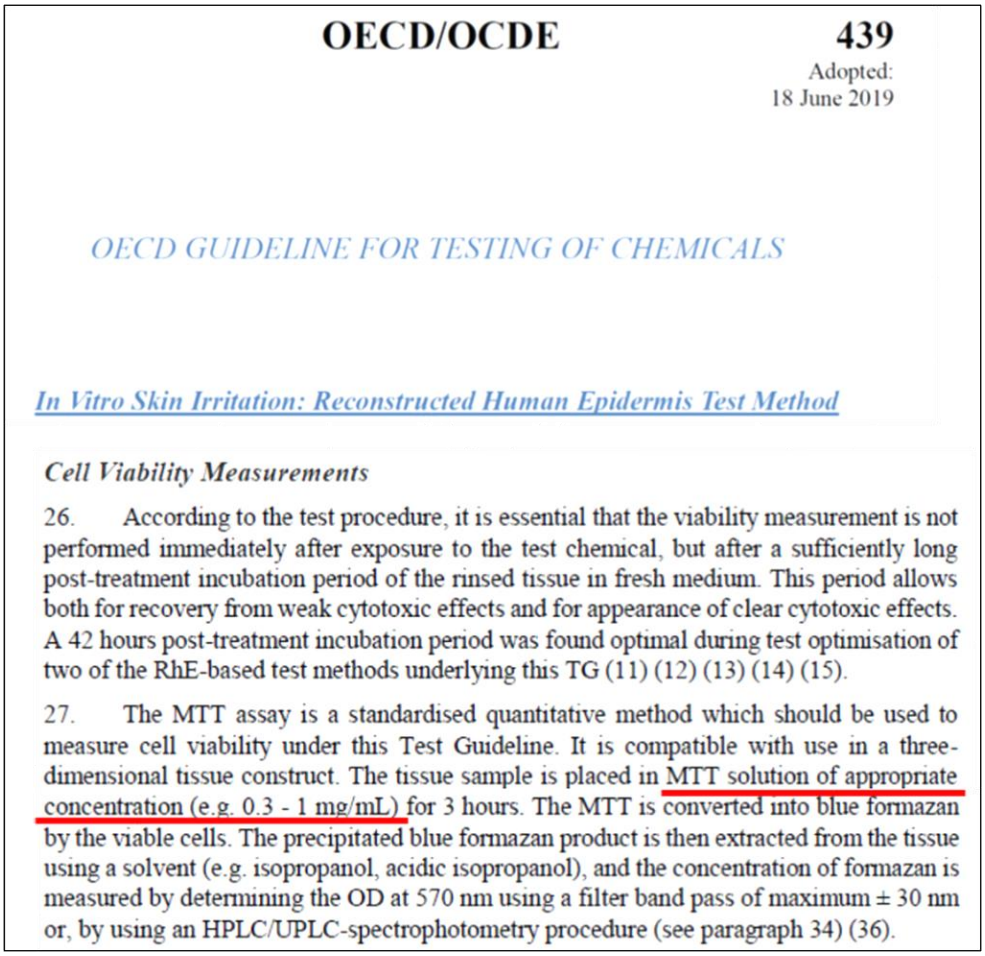
A: Oversea transportation to China was done in the spring season. However, the container was thermostated at room temperature. The shipment box is designed to maintain an internal temperature of 15℃ ~ 25℃. We have attached a report by World Courier, which details the shipment box temperature validation experiment conducted for summer and winter conditions.

Figure 2. Temperature validation results during summer and winter conditions.

4. MTT concentration and Triton X-100 volume used

Q: Can you explain why a lower concentration of MTT solution was used for QC check in the KeraSkin model as compared to the adopted test method i.e., 0.5 vs. 1 mg/mL used for other tissue models of similar size?

A: In Biosolution's QC test, we adopted the concentration recommended by the MTT kit providers (Sigma and Roche). In addition, OECD TG 439 suggests a range of MTT (0.3 to 1.0 mg/mL).



OECD/OCDE 439

Adopted:
18 June 2019

OECD GUIDELINE FOR TESTING OF CHEMICALS

In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method

Cell Viability Measurements

26. According to the test procedure, it is essential that the viability measurement is not performed immediately after exposure to the test chemical, but after a sufficiently long post-treatment incubation period of the rinsed tissue in fresh medium. This period allows both for recovery from weak cytotoxic effects and for appearance of clear cytotoxic effects. A 42 hours post-treatment incubation period was found optimal during test optimisation of two of the RhE-based test methods underlying this TG (11) (12) (13) (14) (15).

27. The MTT assay is a standardised quantitative method which should be used to measure cell viability under this Test Guideline. It is compatible with use in a three-dimensional tissue construct. The tissue sample is placed in MTT solution of appropriate concentration (e.g. 0.3 - 1 mg/mL) for 3 hours. The MTT is converted into blue formazan by the viable cells. The precipitated blue formazan product is then extracted from the tissue using a solvent (e.g. isopropanol, acidic isopropanol), and the concentration of formazan is measured by determining the OD at 570 nm using a filter band pass of maximum ± 30 nm or, by using an HPLC/UPLC-spectrophotometry procedure (see paragraph 34) (36).

Indeed, VRMs and PS based test methods of OECD TG 439 adopt a range of MTT concentrations as shown below,

Table 3. Comparison of MTT concentrations in VRMs

	EpiSkin™	EpiDerm™ SIT	SkinEthic RHE™	LabCyte EPI-MODEL24 SIT	epiCs®	Skin+®
MTT Final conc.	0.3 mg/mL	1 mg/mL	1 mg/mL	0.5 mg/mL	1 mg/mL	0.5 mg/mL

Q: Can you explain why different concentrations of MTT were used for QC vs. SIT (i. e. 0.5 mg/mL vs. 0.4 mg/mL)?

A: The MTT concentration used in the SIT protocol was decided independently from the tissue manufacturer practices. The KeraSkin™ SIT was originally developed by AmorePacific R&D center which determined and set an MTT concentration of 0.4 mg/mL, a minimum

concentration value deemed adequate through a SOP optimization process on the basis of MTT's presumed toxicity. As the model QC and SIT are considered separate procedures, we believe that the difference in MTT concentration between the two does not pose a significant problem.

Q: The PRP members asked if the test developers have evaluated the impact of different concentrations of MTT on tissue viability. Test developers to assess, using the same tissue batch, the ET_{50} values obtained with 0.4 mg/mL, 0.5 mg/mL and 1 mg/mL of MTT, using both 50 and 100 µL of Triton X-100.

A: PRP commented on ET_{50} test conditions with respect to MTT concentration and application volume of Triton X-100. We conducted 5 occasions of tests as shown below,

Table 4. Effects of MTT concentration and application volume on ET_{50} values of QC

#	Application volume	0.4 mg/mL MTT		0.5 mg/mL MTT		1 mg/mL MTT	
		NC OD	ET50(hr)	NC OD	ET50(hr)	NC OD	ET50(hr)
1	50 µL	0.70	8.13	0.85	8.24	1.31	7.09
	100 µL	0.74	8.18	0.87	8.17	1.38	7.01
2	50 µL	0.90	8.59	0.96	8.11	1.52	7.21
	100 µL	0.92	8.28	1.03	8.56	1.47	7.00
3	50 µL	0.94	10.23	1.06	9.30	1.54	8.46
	100 µL	0.98	10.14	1.09	9.46	1.52	8.12
4	50 µL	0.74	8.02	0.88	8.54	1.39	7.00
	100 µL	0.73	8.55	0.87	8.07	1.32	6.94
5	50 µL	0.81	9.11	0.92	9.23	1.43	7.19
	100 µL	0.84	9.25	0.98	9.19	1.37	7.68
Average±SD	**50 µL**	**0.82±0.10**	**8.82±0.90**	**0.93±0.08**	**8.68±0.55**	**1.44±0.10**	**7.39±0.60**
	100 µL	**0.84±0.11**	**8.88±0.82**	**0.97±0.10**	**8.69±0.62**	**1.41±0.08**	**7.35±0.53**

In regards to the ET_{50} measurement method included in KeraSkin's QC category, we determined whether the MTT concentration and Triton X-100 volume had any effect on the resulting values. As a result, it was found that increasing the MTT concentration slightly decreased the ET_{50}, while changes in Triton X-100 did not alter the ET_{50}. From this observation, we think that changing of QC condition is not necessary.

5. Explanation for the 42 hours post-exposure

Q: In the protocol, there are described thirty minutes for exposed period and 42 hours for post-incubation period. The text should be added to explain why this number of hours is used and fixed standard durations.

A: Post-incubation time of 42 hours is the same as the VRMs.

When the test substance was applied according to the Protocol 1.4, the mesh used

previously was removed, and the application method (the amount and the time) of test substance was altered in order to evenly distribute the substance on the surface of the artificial skin.

The amount was increased (30 µL→40 µL) to evenly distribute the substance and the method was optimized by shortening the treating time (45 mins→30 mins).

The viscous substance sometimes let the mesh stay on the skin even after the washing process, so we did not use the mesh in the test. We added this information to the validation report.

6. Re-test of Ferric chloride

Q: Ferric chloride appears once as Cat. 1B/1C (answer to question 13) and another time as Cat. 2 (answer to question 14) in your responses to our comments. Can you clarify which is its classification and its supporting reference?

A: FeCl3 is Cat. 1B/1C (or R34). The supporting reference is shown below,
Kandárová, H., Hayden, P., Klausner, M., Kubilus, J., Kearney, P., Sheasgreen, J. (2009). In vitro skin irritation testing: Improving the sensitivity of the EpiDerm skin irritation test protocol. ATLA 37, 671-689.

Q: Ferric III Chloride - that has been identified as problematic material also in the past – they should try to re-test fresh material. The negative result of this compound could be seen in the past if the sample was aged or improperly stored.

A: We conducted KerSkin™ SIT for FeCl3 (CAS No. 7705-08-0, Solid) three times even though this chemical is removed from the additional chemical list since it is Cat 1.

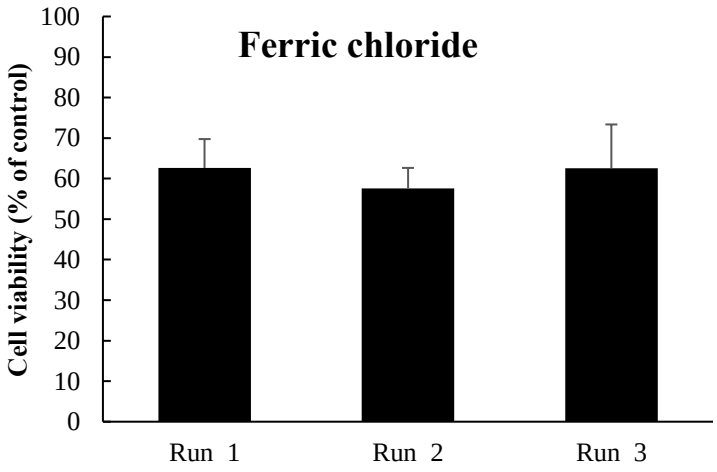


Figure 3. Cell viability of Ferric chloride with MTT assay

Table 5. Cell viability values of Ferric chloride with MTT assay

Chemical	Run # (Date)	Cell viability (%)	
		MEAN	SD
Ferric chloride	Run 1 (2020.05.08)	62.60	7.15
	Run 2 (2020.06.04)	57.58	5.05
	Run 3 (2020.06.17)	62.57	10.79

*Each run was conducted with fresh-opened new bottle.

7. The results of cell viability obtained with different MTT concentrations

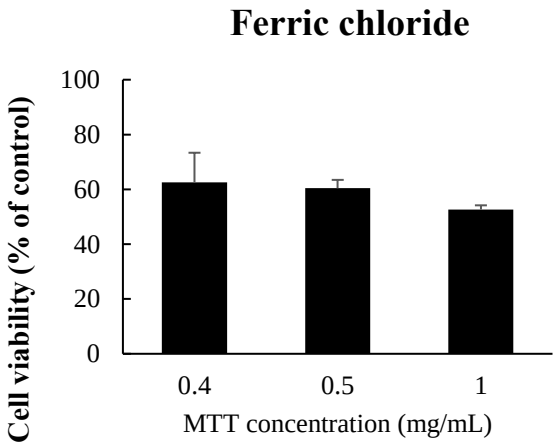


Figure 4. Cell viability of Ferric chloride with different MTT concentrations

Table 6. Cell viability values of Ferric chloride with different MTT concentrations

Chemical	MTT Concentration (mg/mL)	Cell viability (%)	
		MEAN	SD
Ferric chloride	0.4	62.57	10.79
	0.5	60.41	3.05
	1	52.62	1.54

8. QC acceptance criteria

Q: The acceptance range for the NC is too broad. Usually, the range is established as the historical mean +/- 2*SD and (not 3*SDs). Otherwise, all the production would fit into the

guideline, and this could lead to the variation in predictions. Ideally, the group should check with those lots that were used during the validation study.

A: When 2SD was used to set the acceptance range, a total of 14 batches (NC OD 8 batch, ET50 6 batch, IC50 2 batch) were found to be outside the new acceptable range (Figure 5, Table 7), and 7 amongst the 14 unqualifying batches were used in skin irritation tests (Table 8). Notably, 4 batches were used in proficiency tests and reproducibility tests with their results matching with(correspond with) those of the VRMs (Table 9).

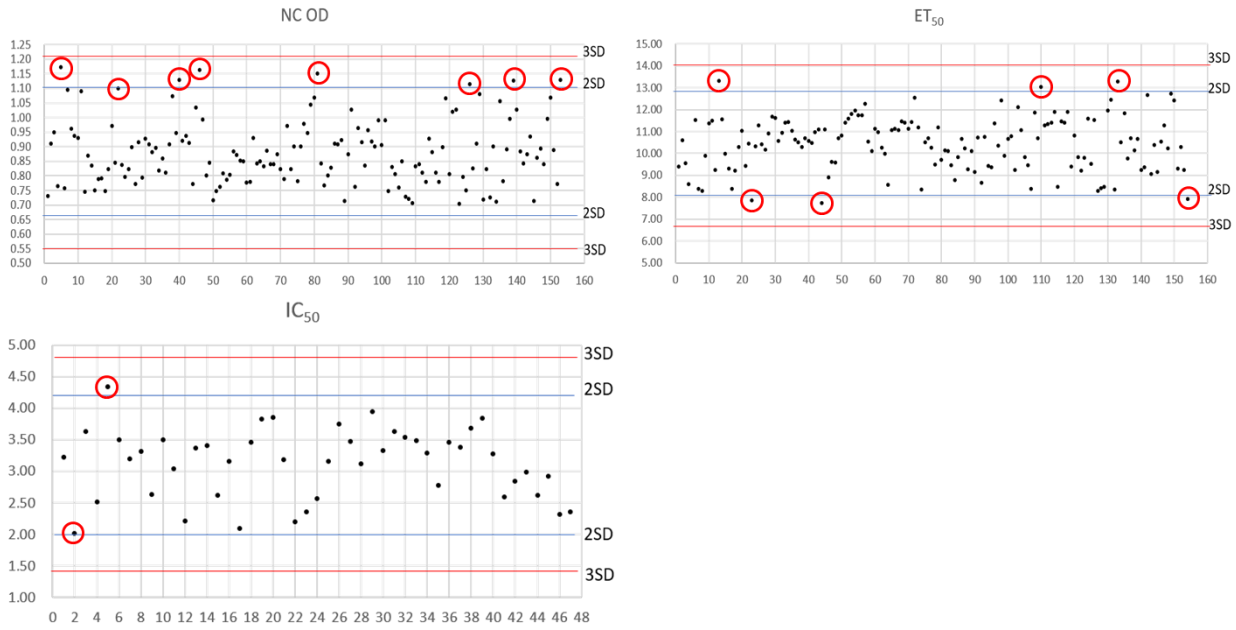


Figure 5. Fourteen of 153 batches would be out of QC (NC OD 8 cases, ET_{50} 6 cases, IC_{50} 2 cases, Multiple 2 cases

)

Table 7. Overview of batches out of ± 2SD spec

batch No.	NC OD (0.66 < OD < 1.10)	ET_{50} (8.05 < ET_{50} < 12.84)	IC_{50} (2.02 < IC_{50} < 4.23)
KS15005	1.17		
KS15012		13.30	
KS16004	1.10	7.85	
KS17001	1.13		
KS17004		7.75	
KS17007	1.16		
KS17042	1.15		
KS18014			2.01
KS18015		13.03	
KS18017			4.34
KS18033	1.12		
KS19004*		13.29	
KS19011*	1.13		
KS19025	1.13	7.91	

*Batches used for the main WLR/BLR reproducibility test

Table 8. Overview of 7 batches out of ± 2SD spec used for KeraSkin™ SIT

	Batch no.	Test used	NC	PC (%)
1	KS15012	Lot test	1.03	9.30
2	KS17004	Lot test	0.97	2.89
3	KS18014	proficiency test (KCL)	0.90	5.45
4	KS18015	proficiency test (BS)	0.72	1.86
5	KS18033	Lot test	1.15	7.04
6	KS19004	reproducibility test (BS)	1.00	3.16
7	KS19011	reproducibility test (KCL)	1.25	3.41
		Mean±SD	1.00±0.27	4.73±2.66
	*Main study 20 batches	Mean±SD	0.98±0.15	4.25±4.19

* 20 batches used for the main WLR/BLR reproducibility test

Table 9. Test results from batches out of ± 2SD spec

No	Chemical	Cas No.	Physical state	UN GHS	KS18014	KS18015	KS19004	KS19011
1	1-Decanol	112-30-1	liquid	Cat 2			8.94	10.18
2	cyclamen aldehyde	103-95-7	liquid	Cat 2	-0.01	-1.89	2.14	2.62
3	1-bromohexane	111-25-1	liquid	Cat 2	14.48	16.04		13.07
4	2-Chloromethyl-3,5-dimethyl-4-methoxy pyridine HCL	86604-75-3	solid	Cat 2			8.03	
5	Di-n-propyl disulphide	629-19-6	liquid	Cat 2				
6	potassium hydroxide(5% aq.)	1310-58-3	liquid	Cat 2	-0.41	0.25	1.80	
7	Benzenethiol, 5-(1,1-dimethylethyl)-2-methyl	7340-90-1	liquid	Cat 2			7.33	0.62
8	1-methyl-3-phenyl-1-piperazine	5271-27-2	solid	Cat 2	0.4	0.01	-0.95	
9	heptanal	111-71-7	liquid	Cat 2	0.59	-0.73	8.41	2.70
10	tetrachloroethylene	127-18-4	liquid	Cat 2			9.45	
11	1-bromo-4-chlorobutane	6940-78-9	liquid	NC			15.85	
12	Diethyl phthalate	84-66-2	liquid	NC				82.17
13	naphthalene acetic acid	86-87-3	solid	NC	88.94	79.95		65.63
14	allyl phenoxy-acetate	7493-74-5	liquid	NC				
15	isopropanol	67-63-0	liquid	NC	94.51	68.88		70.66
16	4-methyl-thio-benzaldehyde	3446-89-7	liquid	NC				5.26
17	methyl stearate	112-61-8	solid	NC	81.25	113.61	91.44	87.20
18	heptyl butyrate	5870-93-9	liquid	NC	77.79	100.67		
19	hexyl salicylate	6259-76-3	liquid	NC	77.67	93.05		
20	cinnamaldehyde	104-55-2	liquid	NC				

Irritant | Non-irritant

9. Range of IC_{50}

Q: The range of the QC parameter IC_{50} differs from those of other similar test methods, e.g. larger IC_{50} range. Is there any explanation why?

A: We think that difference in IC_{50} of KeraSkin™, 1.5 < IC_{50} < 4.7 (mg/mL), is from applying 3-SD spec range. e.g. 1 - 3 (Episkin), 1.4 – 4 (Labcyte)

10. Information of keratinocytes for KeraSkin

Q: It would be good to have more information of keratinocytes for KeraSkin. Which human Keratinocytes have been used for the KeraSkin model? Either individual or pooled Korean cells is used? In addition, the information is unclear the range of ages, sex and normality.

A: Keratinocytes used for the manufacture of KerSkin™ were from the residual skin tissues from circumcision. Donors were male and aged between 10 to 17. One batch was from a single donor and karyotyping was conducted for normality check.

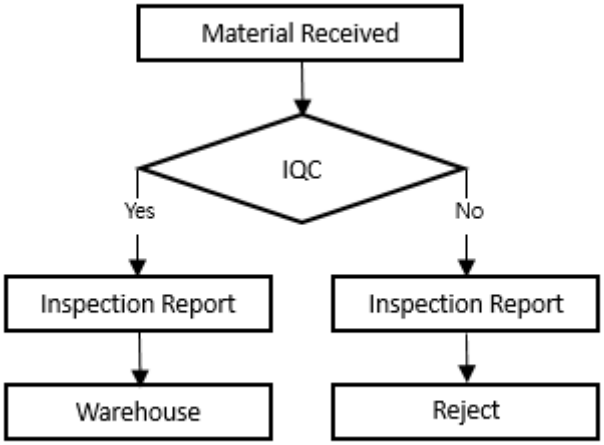
11. Information on the intellectual property rights of KeraSkin

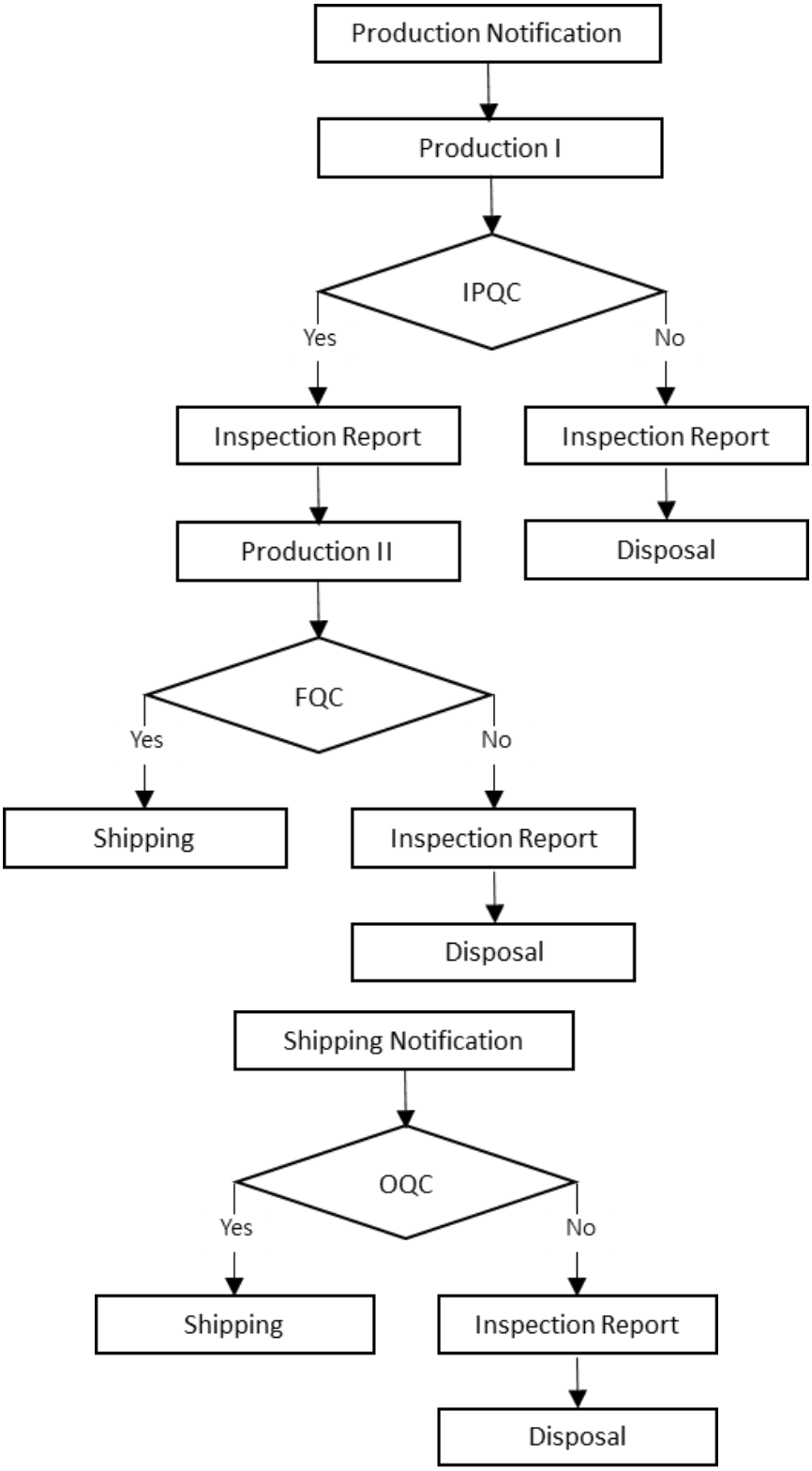
Q: It is necessary the more information on the intellectual property rights of this model.

A: KeraSkin™ is a trademark registered in Korea. There is no IP issue for model manufacture, media, or test method.

12. QA procedure for 3D RhE

Q: If a quick QA procedure for 3D RhE can be presented as a flowchart, such as Figure 2 or Figure 8, it would be very helpful.





*IQC: Incoming Quality Control, IPQC: In-Process Quality Control, FQC: Final Quality Control, OQC: Outgoing Quality Control

Figure 6. A flowchart of QA procedure for KeraSkin™ manufacturing

13. About tissue batches

Q: It was also questioned how many batches were tested during the validation study. Is it possible to have information on which tissue batches were tested by which laboratory during the validation trial?

Table 10. Tissue batches tested by laboratories during the validation study

Test	Laboratory	Batch No.
Proficiency	BS	KS18015
	KCL	KS18014
	COSMAX	KS19006
		KS19007
Predictive Capacity (20 PS)	BS	KS19003
		KS19004
		KS19006
		KS19007
		KS19008
		KS19009
	KCL	KS19003
		KS19005
		KS19007
		KS19008
		KS19010
		KS19011
	COSMAX	KS19009
		KS19010
		KS19011
		KS19013
		KS19019
		KS19020
		KS19022
Predictive Capacity (54 chemicals)	BS	KS19026
		KS19027
		KS19028
		KS19033
	KCL	KS19026
		KS19027
		KS19033

14. Participating laboratories

Q: It was asked why Biotoxtech did not take part to the PS-based validation study.

A: Biotoxtech passed the transferability phase, but they failed the proficiency phase where the 10 proficiency chemicals recommended within OECD TG 439 were tested. In particular, isopropanol was predicted as a false positive, which may be due to issues with their washing procedures.

Q: Why was the COSMAX transferability assessed with four chemicals only?

A: KCL and BTT failed in the 1st in-house trial. Thus, a second trial was conducted with more chemicals. In contrast, COSMAX succeeded in the 1st in-house trial.

Q: All participating laboratories to the validation study are situated in Seoul or its neighbourhoods. Is it possible to provide a statement on the relationship and/or independency between these laboratories?

A: They are separate facilities, not related or affiliated. Biosolution is the manufacturer of KeraSkin™, COSMAX is a cosmetic company (15 km away from Biosolution) and KCL is a GLP CRO (48 km away from Biosolution).

15. *In vivo* references of test chemicals for the predictive capacity assessment

Q: Please provide the information on the sources of *in vivo* data of additional test chemicals.

A: The references are provided as below (Table 11),

1 ***Table 11. Information on the sources of in vivo data of additional 46 chemicals in the validation study of KeraSkin™ SIT***

No	Chemical	CAS no.	In vivo category	In vivo score	Human in vivo result	In vivo class	KeraSkin™ SIT	VRMs				VRMs references
								EpiSkin	SkinEthic	EpiDerm	LabCyte	
1	Nonanoic acid	112-05-0	Cat 2[b]	4.0[b]	I[b]	I[b]	3.0	4.6[*]	1.1	-	-	Tornier et al., 2010; Li et al., 2017
2	Butyl methacrylate	97-88-1	Cat 2[c]	3.0[c]	NC[c]	I[c]	24.6	11.3 (5.7)[*]	1.1-3.7	11.6	24.5-33.6	Kandárová et al., 2009; Alépée et al., 2015b; Li et al., 2017; OECD, 2011
3	Butyric acid	107-92-6	Cat 1B[f]	-	-	I[f]	2.2	2.1-4.5	0.4-1.1	-	-	Alépée et al., 2019; Alépée et al., 2015b
4	Heptanoic acid	111-14-8	R34[g]	- (PII 4.8)[g]	I[g]	I[g]	2.2	-	<20	-	-	Tornier et al., 2006
5	Decanoic acid (Capric acid)	334-48-5	Cat 2[h]	4.0[h]	I[h]	I[h]	43.6	3.1	<20	5.5	6.1-17.6	Tornier et al., 2010; Tornier et al., 2006; Kandárová et al., 2009; OECD, 2011
6	1-Bromopentane	110-53-2	Cat 2[i]	2.7[i] (PII 4.44)[i]	-	I[i]	11.6	31.2 (9.9-45.1)[*]	<20	5.8 (81.7)[※]	17.7-24.3	Kandárová et al., 2009; Li et al., 2017; Tornier et al., 2006; OECD, 2011; OECD, 2010
7	alpha-Terpineol	98-55-5	Cat 2[j]	2.7[j] (PII 4/4.44/4.75)[j]	NC[j]	I[j]	17.1	8.9 (2.8-15.2)[※]	<20	7.1 (17.9-70.9)[※]	7.3-14.5	Kandárová et al., 2009; OECD, 2011; OECD, 2010; Tornier et al., 2006
8	Octanoic acid (Caprylic acid)	124-07-2	Cat 1B/1C[l]	(PII 4.44)[l] (MGP 3.0)[l]	I[l]	I[l]	2.0	4.5-6.1	0.5-1.1	-	-	Alépée et al., 2019; Alépée et al., 2015b; Tornier et al., 2006
9	Polyethylene glycol 400 (PEG-400)	25322-68-3	NC[n]	0[n] (PII 0.0)[n]	NC[n]	NI[n]	96.1	101.4	>80 (93.4)[※※]	99.9	98.2-106.6	OECD, 2011; Kandárová et al., 2009; Sugiyama et al., 2018; Tornier et al., 2006
10	(3-Chloropropyl)trimethoxysilane (Silane A-1430)	2530-87-2	NC[o]	0[o]	-	NI[o]	3.3	36.0-94.6 (10.8)[*]	80.7	61.7-98.6	-	Alépée et al., 2015b; Li et al., 2017; Alépée et al., 2019; Tornier et al., 2010
11	3,3′-Dithiodipropionic acid	1119-62-6	NC[p]	0[p] (PII 0)[p]	-	NI[p]	96.3	96.7-107.5	102.4-117.1	98.0	89.9-100	Alépée et al., 2019; Alépée et al., 2015b; Kandárová et al., 2009; OECD, 2011

No	Chemical	CAS no.	In vivo category	In vivo score	Human in vivo result	In vivo class	KeraSkin™ SIT	VRMs				VRMs references
								EpiSkin	SkinEthic	EpiDerm	LabCyte	
12	2-Phenylethanol (Phenylethyl alcohol)	60-12-8	NC[q]	1.0[q] (PII 0.92/2.22)[q]	-	NI[q]	0.3	63.1-104.4 (91.7)[*]	5.6	45.7-92.9	-	Tornier et al., 2010; Alépée et al., 2019; Li et al., 2017; OECD, 2010
13	Benzyl salicylate	118-58-1	NC[r]	0.3[r] (PII 0.33)[r]	NC[r]	NI	98.5	121.37 (96.4)[*]	86.5-113.3	89.5	93.6-99.9	Kandárová et al., 2009; Li et al., 2017; OECD, 2011; Alépée et al., 2015b
14	1-(4-Chlorophenyl)-3-(3,4-dichlorophenyl)urea	101-20-2	NC[s]	-	-	NI[s]	102.3	111.2	-	108.2	-	Alépée et al., 2015a; ECHA, 2020b
15	3,3-Dimethylpentane	562-49-2	NC[t]	0[t] (PII 0)[t]	-	NI[t]	88.3	-	-	102.4	72.3-90.8	Kandárová et al., 2009; OECD, 2011;
16	4,4′-Methylenebis(2,6-di-tert-butylphenol)	118-82-1	NC[u]	0[u] (PII 0)[u]	-	NI[u]	97.3	85.8-111.2	86.5-113.3	96.9	100.0-101.3	Alépée et al., 2019; Alépée et al., 2015b; OECD, 2011; Kandárová et al., 2009
17	Dodecanoic acid (Lauric acid)	143-07-7	NC[r]	0.3[r] (PII 0.44)[r]	NC[r]	NI[r]	84.8	102.5-127.6	78.0-102.1	20.2	94.0-110.0	Alépée et al., 2015b; Alépée et al., 2019; Kandárová et al., 2009; OECD, 2011
18	1-Chloro-3-nitrobenzene (3-Chloronitrobenzene)	121-73-3	NC[v]	0[v] (PII 0)[v]	-	NI[v]	87.7	90.0 (102.5)[*]	-	96.9	95.2-104.3	Kandárová et al., 2009; OECD, 2011; Li et al., 2017
19	Benzyl benzoate	120-51-4	NC[v]	0[v] (PII 0 / 1.58)[v]	-	NI[v]	98.3	110.0	84.3-104.1	93.4	99.6-105.7	Kandárová et al., 2009; OECD, 2011; Alépée et al., 2015b
20	2-(Formylamino)-3-thiophenecarboxylic acid	43028-69-9	NC[w]	0[w]	-	NI[w]	100.9	89.0-95.8	108.0	97.8-105.7	-	Tornier et al., 2010; OECD, 2010
21	Sodium bisulphite	7631-90-5	NC[v]	1.0[v] (PII 1.0)[v]	-	NI[v]	9.4	108.0	79.1-99.7	56.1	11.1-74.7	Kandárová et al., 2009; Alépée et al., 2015b; OECD, 2011
22	4-Acetoxy-2,5-dimethyl-3(2H)furanone (2,5-Dimethyl-4-oxo-4,5-dihydrofuran-3-yl acetate)	4166-20-5	NC[w]	0[w]	-	NI[w]	4.6	98.2-114.3	102.3	80.3-91.2	-	Tornier et al., 2010; OECD, 2010

No	Chemical	CAS no.	In vivo category	In vivo score	Human in vivo result	In vivo class	KeraSkin™ SIT	VRMs				VRMs references
								EpiSkin	SkinEthic	EpiDerm	LabCyte	
23	4-Amino-4H-1,2,4-triazole (4-Amino-1,2,4-triazole)	584-13-4	NC[x]	0[x] (PII 0)[x]	-	NI[x]	89.7	98.3	102.2-106.0	91.0	97.4-101.0	Kandárová et al., 2009; OECD, 2011; Alépée et al., 2015b
24	Di-propylene glycol	25265-71-8	NC[y]	0[y] (PII 0.3)[y]	NC[y]	NI[y]	94.9	81.5-111.0	95.9-103.5	93.5	92.9-109.9	Kandárová et al., 2009; Alépée et al., 2015b; Alépée et al., 2019; OECD, 2011
25	Di(propylene glycol) butyl ether, mixture of isomers (Dipropylene glycol monobutyl ether; DPnB)	29911-28-2	NC[w]	0[w]	-	NI[w]	13.9	85.0-113.9	97.4	101.2	-	Alépée et al., 2015b; Tornier et al., 2010; Alépée et al., 2019
26	Erucamide	112-84-5	NC[x]	0[x] (PII 0)[x]	-	NI[x]	103.1	97.8-106.2	95.1-107.7	102.7	90.0-102.5	Kandárová et al., 2009; OECD, 2011; Alépée et al., 2015b; Alépée et al., 2019
27	Propylene glycol	57-55-6	NC[z]	- (PII 0.5)[z]	NC[z]	NI[z]	84.5	-	-	-	-	-
28	Triethylene glycol	112-27-6	NC[w]	0[w]	-	NI[w]	76.6	91.7-116.9 (101.9)[*]	101.4	95.0-95.3	-	Alépée et al., 2015b; Alépée et al., 2019; OECD, 2010; Li et al., 2017
29	Sodium bicarbonate	144-55-8	NC[x]	0[x] (PII 0.11)[x]	-	NI[x]	91.0	92.0-104.2	94.7-113.3	90.9	99.6-100.3	Kandárová et al., 2009; Alépée et al., 2019; Alépée et al., 2015b; OECD, 2011
30	Isopropyl palmitate	142-91-6	NC[x]	1.0[x] (PII 1.44)[x]	NC[x]	NI[x]	106.0	104.3	100.7-115.1	93.0	102.5-115.9	Kandárová et al., 2009; Alépée et al., 2015b; OECD, 2011
31	Isopropyl myristate	110-27-0	NC[x]	1.0[x] (PII 1.22)[x]	NC[x]	NI[x]	101.9	127.4	98.6-110.5	97.5	97.2-107.9	Kandárová et al., 2009; Alépée et al., 2015b; OECD, 2011
32	2-Ethylhexyl 4-methoxycinnamate (Octinoxate)	5466-77-3	NC[aa]	-	-	NI[aa]	106.5	-	83.3-109.7	-	-	Molinari et al., 2013
33	Tetrabromophenol blue	4430-25-5	NC[ab]	-	-	NI[ab]	89.9	109.8 (110.8)[※※※]	-	-	-	Alépée et al., 2015a; Alépée et al., 2016;
34	Piroctone olamine	68890-66-4	Cat 2[m]	-	-	I[m]	1.7	6.0 (7-11)[**]	-	-	-	Alépée et al., 2016; SCCS, 2010

No	Chemical	CAS no.	In vivo category	In vivo score	Human in vivo result	In vivo class	KeraSkin™ SIT	VRMs				VRMs references
								EpiSkin	SkinEthic	EpiDerm	LabCyte	
35	25% Cetyltrimethylammonium chloride solution (in D.W.) (Cetrimonium chloride)	112-02-7	Cat 2[ae]	-	-	I[ae]	1.1	10.5	-	-	-	Alépée et al., 2016;
36	Iron (III) chloride (Ferric chloride)	7705-08-0	R34[af]	-	-	I[af]	57.7	-	-	12.5[***]	-	Kandárová et al., 2009
37	2-Hydroxy-4-methoxybenzophenone, (Benzophenone-3)	131-57-7	NC[ah]	-	-	NI[ah]	99.3	104.1 (64-119)[**]	-	-	-	Alépée et al., 2016; SCCS, 2010
38	Cyclohexadecanone	2550-52-9	NC[ai]	0[ai]	-	NI[ai]	103.9	112.4-121.9	-	7.9-113.6	-	OECD, 2010
39	Methyl laurate	111-82-0	Cat 3[aj]	2.0[aj] (PII 3.89)[aj]	NC[aj]	NI[aj]	91.2	83.9[*]	>80	103.3	-	OECD, 2010; Li et al., 2017; Tornier et al., 2006
40	Capryl-isostearate	209802-43-7	NC[ai]	1.0[ai]	-	NI [ai]	94.1	96.0-102.3	-	99.5-108.0	-	OECD, 2010
41	2-Ethylhexyl-4-aminobenzoate	26218-04-2	NC[ai]	0.7[ai]	-	NI[ai]	103.1	90.9-111.4	-	91.9-107.3	-	OECD, 2010
42	2-Phenylhexanenitrile	3508-98-3	Cat 3[ai]	1.7[ai]	-	NI[ai]	76.0	86.7-116.2	-	73.9-82.1	-	OECD, 2010
43	Barium sulfate	7727-43-7	NC[ak]	-	-	NI[ak]	94.8	95.3	99.4	99.0	92.9	Sugiyama et al., 2018
44	Diisopropyl sebacate	7491-02-3	NC[al]	-	-	NI[al]	93.8	91.1	91.0	-	104.6	Sugiyama et al., 2018
45	10% Xanthan gum (in D.W.)	11138-66-2	NC[am]	-	-	NI[am]	99.6	101.3	98.4	-	94.0	Sugiyama et al., 2018
46	3-Chloro-4-fluoronitrobenzene	350-30-1	NC[an]	1.0[an] (PII 1.67)[an]	-	NI[an]	80.7	11.0-54.9 (5.0)[*]	101.6	53.2-101.7	-	OECD, 2010; Tornier et al., 2010; Li et al., 2017

VRMs: Validated Reference Methods; EpiSkin: EpiSkin™ SIT; SkinEthic: SkinEthic™ RHE; EpiDerm: EpiDerm™ SIT; LabCyte: LabCyte EPI-MODEL 24 SIT.

Definitions of the table abbreviations.

VRMs = In vitro cell viability value, % of control; NC = No Category, skin non-irritant; Cat 2 = UN GHS Category 2, skin irritant.

Cat 3 = UN GHS Optional Category 3, skin mild irritant ※OECD TG 439(In vitro skin irritation: RhE test methods) is not allow the classification of chemicals to the optional category 3. Under this test guideline, Cat 3 is considered as No Category.

[*] EpiSkin data produced in China; [**] SCCS Memorandum (addendum) data on the in vitro EpiSkin; ※[OECD, 2010] data; ※※[Sugiyama et al., 2018] data; ※※※[Alépée et al., 2016] data.

[b] In vivo data source: Jirova et al., 2010; Li et al., 2017; Basketter et al., 2012.
[c] In vivo data source: Jirova et al., 2010; Kandárová et al., 2009; Li et al., 2017; Tornier et al., 2010; Alépée et al., 2015b.
[f] In vivo data source: Alépée et al., 2019; Alépée et al., 2015b.
[g] In vivo data source: Golla et al., 2009; Tornier et al., 2006.
[h] In vivo data source: Kandárová et al., 2009; Jirova et al., 2010; OECD, 2011.
[i] In vivo data source: Kandárová et al., 2009; ECETOC, 1995; OECD, 2010.
[j] In vivo data source: Kandárová et al., 2009; ECETOC, 1995; Jirova et al., 2010; OECD, 2010.
[l] In vivo data source: ECETOC, 1995; Jacobs et al., 2000; Alépée et al., 2019.
[m] In vivo data source: Alépée et al., 2016; SCCS, 2010; ECHA, 2020e.
[n] In vivo data source: Kandárová et al., 2009; OECD, 2011; Basketter et al., 2012; Tornier et al., 2006.
[o] In vivo data source: Tornier et al., 2010; OECD, 2010; Alépée et al., 2015b.
[p] In vivo data source: Kandárová et al., 2009; ECETOC, 1995; OECD, 2011; OECD, 2010.
[q] In vivo data source: Tornier et al., 2010; ECETOC, 1995; OECD, 2010.
[r] In vivo data source: Kandárová et al., 2009; ECETOC, 1995; Basketter et al., 2012.
[s] In vivo data source: Alépée et al., 2015a; ECHA, 2020b.
[t] In vivo data source: Kandárová et al., 2009; OECD, 2011.
[u] In vivo data source: OECD, 2011; ECETOC, 1995; Kandárová et al., 2009.
[v] In vivo data source: ECETOC, 1995; Kandárová et al., 2009.
[w] In vivo data source: Tornier et al., 2010; Hoffmann et al., 2008; OECD, 2010.
[x] In vivo data source: ECETOC, 1995; Kandárová et al., 2009; OECD, 2010; Hoffmann et al., 2008.
[y] In vivo data source: ECETOC, 1995; Kandárová et al., 2009; Jirova et al., 2010; Tornier et al., 2010; Hoffmann et al., 2008.
[z] In vivo data source: Basketter et al., 2012; Golla et al., 2009; ECHA, 2020c.
[aa] In vivo data source: Molinari et al., 2013; SCC, 1999.
[ab] In vivo data source: Alépée et al., 2015a; Alépée et al., 2016; SCCS, 2019.
[ac] In vivo data source: Alépée et al., 2016; SCCS, 2010; SCCS, 2009; Becker et al., 2012; CIR, 1997; IMAP, 2015.
[af] In vivo data source: OECD, 2010; ECHA, 2020f; ECHA, 2020g; Kandárová et al., 2009.
[ah] In vivo data source: Alépée et al., 2016; Mikkelsen et al., 2015; SCCP, 2006; ECHA, 2020i.
[ai] In vivo data source: Hoffmann et al., 2008; OECD, 2010
[aj] In vivo data source: ECETOC, 1995; Hoffmann et al., 2008; Basketter et al., 2012; OECD, 2010.
[ak] In vivo data source: ECHA, 2020j; CIR, 2014.
[al] In vivo data source: ECHA, 2020k; Fiume et al., 2012.
[am] In vivo data source: Fiume et al., 2016; JECFA, 1987; Sugiyama et al., 2018
[an] In vivo data source: ECETOC, 1995; Hoffmann et al., 2008; OECD, 2010; Tornier et al., 2010

References

Alépée, N., Grandidier, M.-H., Cotovio, J., 2019. Usefulness of the EpiSkin™ reconstructed human epidermis model within Integrated Approaches on Testing and Assessment (IATA) for skin corrosion and irritation. Toxicol. in Vitro 54, 147-167

Alépée, N., Hibatallah, J., Klaric, M., Mewes, K.R., Pfannenbecker, U., McNamee, P., 2016. Assessment of cosmetic ingredients in the in vitro reconstructed human epidermis test method EpiSkin™ using HPLC/UPLC-spectrophotometry in the MTT-reduction assay. Toxicol. in Vitro 33, 105-117

Alépée, N., Barroso, J., de Smedt, A., de Wever, B., Hibatallah, J., Klaric, M., Mewes, K.R., Millet, M., Pfannenbecker, U., Tailhardat, M., Templier, M,, McNamee, P., 2015a. Use of HPLC/UPLC-spectrophotometry for detection of formazan in in vitro reconstructed human tissue (RhT)-based test methods employing the MTT-redction assay to expand thire applicability to strongly coloured test chemicals. Toxicol. in Vitro 29, 741-761

Alépée, N., Grandidier, M.-H., Tornier, C., Cotovio, J., 2015b. An integrated testing strategy for in vitro skin corrosion and irritation assessment using SkinEthic™ Reconstructed Human Epidermis. Toxicol. in Vitro 29, 1779-1792.

Basketter, D., Jírova, D., Kandárová, H., 2012. Review of skin irritation/corrosion hazards on the basis of huamn data: A regulatory perspective. Interdiscip. Toxicol. 5, 98-104.

Becker, L.C., Bergfeld, W.F., Belsito, D.V., Hill, R.A., Klaassen, C.D., Liebler, D., Marks Jr, J.G., Shank, R.C., Slaga, T.J., Snyder, P.W., Andersen, F.A., 2012. Safety assessment of trimoniums as used in cosmetics. Int. J. Toxicol. 32(Supplement 3), 296S-341S.

CIR, 2014. Safety assessment of barium sulfate as used in cosmetics. Final report June 26, 2014. Cosmetic Ingredient Review. https://www.cir-safety.org/sites/default/files/barium062014final.pdf.

CIR, 1997. Final report on the safety assessment of cetrimonium chloride, cetrimonium bromide, and steartrimonium chloride. J. Am. Coll. Toxicol., 16, 195-220.

ECETOC, 1995. Skin irritation and corrosion: Reference chemicals data bank. Technical Report No. 66. European Centre for Ecotoxicology and Toxicology of Chemicals, Brussels Mar. 1995. ISSN 0773-8072-66. http://www.ecetoc.org/wp-content/uploads/2014/08/ECETOC-TR-066.pdf.

ECHA, 2020b. Disseminated Registration Dossier for Triclocarban(CAS No. 101-20-2): Irritation/corrosion_Endpoint summary. European Chemicals Agency. https://echa.europa.eu/registration-dossier/-/registered-dossier/12075/7/4/1.

ECHA, 2020c. Disseminated Registration Dossier for Propane-1,2-diol(CAS No. 57-55-6): Irritation/corrosion_Endpoint summary. European Chemicals Agency. https://echa.europa.eu/registration-dossier/-/registered-dossier/16001/7/4/1.

ECHA, 2020e. Disseminated Registration Dossier for 1-hydroxy-4-methyl-6-(2,4,4-trimethylpentyl)pyridin-2(1H)-one, compound with 2-aminoethanol(1:1) (CAS No. 68890-66-4): Skin irritation/corrosion_001 Key/Experimental result. European Chemicals Agency. https://echa.europa.eu/registration-dossier/-/registered-dossier/5490/7/4/2/?documentUUID=a4754038-8d7d-4f34-8461-b3ccda0ee9b7.

ECHA, 2020i. Disseminated Registration Dossier for Oxybenzone(CAS No. 131-57-7): Irritation/corrosion_Endpoint summary. European Chemicals Agency. https://echa.europa.eu/registration-dossier/-/registered-dossier/5515/7/4/1.

ECHA, 2020j. Disseminated Registration Dossier for Barium sulfate(CAS No. 7727-43-7): Irritation/corrosion_Endpoint summary. European Chemicals Agency. https://echa.europa.eu/registration-dossier/-/registered-dossier/15235/7/4/1.

ECHA, 2020k. Disseminated Registration Dossier for Diisopropyl sebacate(CAS No. 7491-02-3): Irritation/corrosion_Endpoint summary. European Chemicals Agency. https://echa.europa.eu/registration-dossier/-/registered-dossier/10094/7/4/1.

Fiume, M.M., Heldreth, B., Bergfeld, W.F., Belsito, D.V., Hill, R.A., Klaassen, C.D., Liebler, D.C., Marks Jr, J.G. Shank, R.C., Slaga, T.J., Snyder, P.W., Andersen, F.A., 2016. Safety assessment of microbial polysaccharide gums as used in cosmetics. Int. J. Toxicol. 35(Supplement 1), 5S-49S.

Fiume, M.M., Eldreth, H., Bergfeld, W.F., Belsito, D.V., Hill, R.A., Klaassen, C.D., Liebler, D., Marks Jr, J.G., Shank, R.C., Slaga, T.J., Snyder, P.W., Andersen, F.A., 2012. Final report of the cosmetic ingredient review expert panel on the safety assessment of dicarboxylic acid, salts, and esters. Int. J. Toxicol. 31(Supplement 1), 5S-76S.

Golla, S., Madihally, S., Robinson, R.L., Gasem, K.AM., 2009. Quantitative structure-property relationships modeling of skin irritation. Toxicol. in Vitro 23, 176-184.

Hoffmann, S., Saliner, A.G., Patlewicz, G., Eskes, C., Zuang, V., Worth, A.P., 2008. A feasibility study developing an integrated testing strategy assessing skin irritation potential of chemicals. Toxicol. Letters 180, 9-20.

IMAP, 2015. Cationic surfactants: Human health tier II assessment. IMAP Group Assessment Report 03 July 2015, Department of Health National Industrial Chemicals Notification and Assessment Scheme(NICNAS). https://www.nicnas.gov.au/chemical-information/imap-assessments/imap-group-assessment-report?assessment_id=1119#cas-A_8001-54-5.

JECFA, 1987. Toxicological evaluation of certain food additives and contaminants: WHO Food Additives Series 21_Xanthan gum. World Health Organization, Cambridge University Press, 1989. http://www.inchem.org/documents/jecfa/jecmono/v21je13.htm.

Jírová, D., Basketter, D., Liebsch, M., Bendová, H., Kejlová, K., Marriott, M., Kandárová, H., 2010. Comparison of human skin irritation patch test data with in vitro skin irritation assays and animal data. Contact Dermatitis 62, 109-116.

Kandárová, H., Hayden, P., Klausner, M., Kubilus, J., Kearney, P., Sheasgreen, J., 2009. In vitro skin irritation testing: Improving the sensitivity of the EpiDerm skin irritation test protocol. ATLA 37, 671-689.

Li, N., Liu, Y., Qiu, J., Zhong, L., Alépée N, Cotovio, J., Cai, Z., 2017. In vitro skin irritation assessment becomes a reality in China using a reconstructed human epidermis test method. Toxicol. in Vitro 41, 159-167

Mikkelsen, S.H., Lassen, C., Warning, M., Hansen, E., Brinch, A., Brooke, D., Crookes, M., Nielsen, E., Bredsdorff, L., 2015. Survey and health assessment of UV filters. Survey of chemmical substances in consumer products No. 142, 2015. The Danish Environmental Protection Agency, ISBN 978-87-93352-82-7.

Molinari, J., Eskes, C., Andres, E., Remoué, N., Sá-Rocha, V.M., Hurtado, S.P., Barrichello, C., 2013. Improved procedures for in vitro skin irritation testing of sticky and greasy natural botanicals. Toxicol. in Vitro 27, 441-450

OECD, 2011. Peer review report of the validation of the skin irritation test using LabCyte EPI-Model 24. Series on Testing and Assessment No. 155 ENV/JM/MONO(2011)44. http://www.oecd.org/env/ehs/testing/49203490.pdf.

OECD, 2010. Explanatory background document to the OECD test guideline on in vitro skin irritation testing. Series on Testing and Assessment No. 137 ENV/JM/MONO(2010)36. http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2010)36&doclanguage=en.

SCC, 1999. Reports of the Scientific Committee on Cosmetology (ninth series): 2-Ethylhexl-4-methoxycinnamate (CAS No. 5466-77-3). Office for Official Publications of the European Communities 1999_European Communities 2000, ISSN 92-828-8951-3. https://ec.europa.eu/health/scientific_committees/consumer_safety/docs/scc_o_9.pdf.

SCCP, 2006. Opinion on benzophenone-3 COLIPA nºS38. SCCP/1069/06, Opinion adopted by the SCCP during the 10th plenary of 19 December 2006. Scientific Committee on Consumer Products. https://ec.europa.eu/health/archive/ph_risk/committees/04_sccp/docs/sccp_o_078.pdf.

SCCS, 2019. Opinion on tetrabromophenol, 4,4'-(4,5,6,7-tetrabromo-1,1-dioxido-3H-2,1-benzoxathiol-3-yliden)bis-2,6-dibromophenol (C183): Submission IV. SCCS/1610/19, The SCCS adopted the final opinion by written procedure on 13 December 2019. Scientific Committee on Consumer Safety. https://ec.europa.eu/health/sites/health/files/scientific_committees/consumer_safety/docs/sccs_o_232.pdf.

SCCS, 2010. Memorandum (addendum) on the in vitro test EPISKIN™ for skin irritation testing. Scientific Committee on Consumer Safety, SCCS/1392/10, European Union 2010, ISSN 1831-4767. https://ec.europa.eu/health/sites/health/files/scientific_committees/consumer_safety/docs/sccs_s_003.pdf.

SCCS, 2009. Opinion on alkyl (C_{16}, C_{18}, C_{22}) trimethylammonium chloride: For other uses than as a preservative COLIPA n°P72. SCCS/1246/09, The SCCS adopted this opinion at its 5th plenary on 8 December 2009. Scientific Committee on Consumer Safety. https://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_012.pdf.

Sugiyama, M., Akita, M., Alépée, N., Fujishiro, M., Hagino, S., Handa, Y., Ikeda, H., Imai, N., Jitsukawa, S., Katoh, M., Kurihara, K., Kyotani, D., Nomura, S., Okamoto, Y., Okumura, H., Omori, T., Sugibayashi, K., Todo, H., Toyoda, A., Ohno, Y., 2018. Comparative assessment of 24-hr primary skin irritation test and human patch test data with in vitro skin irritation tests according to OECD Test Guideline 439 (for quasi-drugs in Japan). J. Toxicol. Sci. 43, 751-768.

Tornier, C., Amsellem, C., de Brugerolle de Fraissinette, A., Alépée, N., 2010. Assessment of the optimized SkinEthic™ reconstructed human epidermis (RHE) 42 bis skin irritation protocol over 39 test substances. Toxicol. in Vitro 24, 245-256.

Tornier, C., Rosdy, M., Maibach, H.I., 2006. In vitro skin irritation testing on reconstituted human epidermis: Reproducibility for 50 chemicals tested with two protocols. Toxicol. in Vitro 20, 401-416.

PART 2

Independent Peer-Review Panel Report

on the scientific validity of the

KeraSkin™ Skin Irritation Test
Reconstructed human Epidermis method

as a similar test according to OECD Test Guideline 439
and Guidance Document 220

29 July 2020

Table of Contents

I. Summary

The KeraSkin™ Skin Irritation Test (SIT) Reconstructed human Epidermis (RhE) method has been developed as a similar method to the methods currently accepted within OECD Test Guideline (TG) 439 (OECD, 2019) for *in vitro* skin irritation testing. A Performance Standard (PS)- based validation study was conducted from June 2018 to December 2019 (Anonym, 2020) coordinated by the Korean Centre for the Validation of Alternative Methods (KoCVAM) and according to the OECD Guidance Document (GD) 220 and OECD GD 34 (OECD, 2015; OECD, 2005). The method and its PS-based validation study then underwent an independent peer-review between February and July 2020, conducted by an international panel including representatives from other national validation bodies. A total of 12 criteria were evaluated by the Peer Review Panel (PRP) to assess the scientific validity of the KeraSkin™ SIT RhE method as a similar method to the methods falling within the OECD TG 439 according to the minimum performances required within the OECD GD 220.

The PRP agreed that the description of the test method and its Standard Operating Procedure were sufficient and adequate and that the KeraSkin™ SIT RhE method adequately adhered to the essential test method components as required according to the OECD GD 220. The KeraSkin™ SIT RhE method met the study acceptance requirements having 100% complete run sequences per laboratory and 100% over the three laboratories (versus ≥ 85% and ≥ 90% target values required within the OECD GD 220).

The KeraSkin™ SIT RhE method within- (100%, 100%, 95% in the individual laboratories) and between- (100%) laboratory reproducibility obtained with the 20 PS reference chemicals also met the minimum reproducibility requirements of the OECD GD 220 (i.e., ≥ 90% and ≥ 80% respectively). Similarly, the predictive capacity obtained with the 20 PS reference chemicals met the minimum target values required within the OECD GD 220. A sensitivity of 100% (n=10), a specificity of 70% (n=10) and an accuracy of 85% (n=20) were obtained (versus the target values of ≥ 80%, ≥ 70% and ≥ 75% respectively). When combined together with the results obtained by testing 42 additional chemicals in a blind manner a sensitivity of 100.0% (17/17), a specificity of 82.2% (37/45) and an accuracy of 87.1% (54/62) were found for a total dataset of 62 chemicals (distributed as 17 UN GHS Category 2, 5 UN GHS Category 3 and 40 UN GHS No Category). Finally, based on the information available, the applicability domain of the KeraSkin™ SIT RhE method is expected to be similar to the validated reference methods.

Based on the above observations, the PRP concluded that the information provided by KoCVAM and by the test method developers is sufficient and adequate to support the scientific validity of the KeraSkin™ SIT RhE method as a similar method to the methods currently accepted under OECD TG 439 and that it has met the minimum performance standards required within the OECD GD 220, including its essential test method components, study acceptance criteria, reproducibility and predictive capacity.

II. Background and peer-review process

The KeraSkin™ SIT RhE method has been developed as a similar assay to the test methods currently accepted within OECD TG 439 for *in vitro* skin irritation testing based on RhE test methods (OECD, 2019). The method underwent a PS-based validation study in accordance to the recommendations from the OECD GD 220 which establishes performance standards for the assessment of proposed similar or modified *in vitro* RhE test methods for skin irritation testing as described in TG 439 (OECD, 2015). The PS-based validation

study was coordinated by a validation management team designated by KoCVAM and in accordance with the requirements of OECD GD 34 on the validation and international acceptance of new or updated test methods for hazard assessment (OECD, 2005). The PS-based study took place between June 2018 and December 2019 and involved three independent laboratories.

The PS-based validation study of the KeraSkin™ SIT RhE method and its scientific validity were then assessed by an international Peer Review Panel between February and July 2020. The peer-review panel members were designated by KoCVAM and comprised independent experts and members from validation bodies as listed below. The non-governmental expert PRP members provided a declaration of interest as to ensure no conflicts of interest existed. These declaration of interests are available upon request.

- Dave Allen, ILS-NICEATM, USA.
- Ok-Nam Bae, Hanyang University, Republic of Korea.
- Chantra Eskes, Switzerland (chair).
- Helena Kandárova, Slovak Academy of Sciences, Slovakia.
- Sebastian Hoffmann, seh consulting + services, Germany.
- Hajime Kojima, JaCVAM, Japan.

The draft validation study report was provided to the PRP in February 2020 and was later revised based on the comments made by the PRP (Anonym, 2020). The process was coordinated by Chantra Eskes as the PRP chair. Overall, a total of five teleconferences took place during the peer-review of KeraSkin™ SIT RhE method as described below.

31 March 2020	PRP + test developers: Presentation by the test method developers and preliminary questions from the PRP.
28 April 2020	PRP only: PRP draft evaluation in the absence of the test method developers taking into account the answers to the written comments from the PRP.
12 May 2020	PRP + test developers: Responses from test method developers to additional questions raised by the PRP.
12 May 2020	PRP only: Updates of the PRP draft evaluation based on the answers provided by the test developers.
18 June 2020	PRP only: Finalization of the PRP evaluation of the KeraSkin™ SIT RhE method.

In July 2020, a final revised validation report was provided to the PRP and the present report was finalized.

III. Peer-review panel evaluation

The PRP agreed to evaluate the KeraSkin™ SIT RhE method as a similar method to those accepted within OECD TG 439 (OECD, 2019) based on the recommendations from OECD GD 220 (OECD, 2015). The evaluation criteria and their assessments were discussed and agreed by all PRP members.

1. Rationale for the test method

Rationale for the KeraSkin™ SIT RhE method, including a description of the advantages of the test method in terms of i) mechanistic advantages, applicability, predictive capacity, technical advances, reduction in hazardous reagents, ii) IP rights, geographical availability and animal welfare, iii) costs, analysis time, sample amount, competitiveness, iv) others.

The PRP agreed that the description of the test method provided in the validation report was sufficient and adequate. In particular, the PRP was of the opinion that KeraSkin™ SIT RhE method presents logistic and practical advantages as compared to the currently accepted test methods regarding e.g., its geographical availability, costs and transportation time.

2. Test method protocol

A detailed protocol for the KeraSkin™ SIT RhE method should be available.

A Standard Operating Procedure (SOP) on the KeraSkin™ SIT RhE method was made available to the PRP including its historical updates. The PRP agreed that the information provided was sufficient and adequate. Furthermore, the PRP acknowledged the inclusion of the Quality Control (QC) procedures for the KeraSkin™ RhE model as an annex to its SOP, increasing transparency and allowing end-users to compare these procedures to the QC procedures of other accepted test methods.

3. Adherence to the essential test method components

Adherence to the essential test method components as described in paragraphs 6 to 23 of GD 220 should be demonstrated for the KeraSkin™ SIT RhE method regarding i.e., the general conditions, the functional conditions and the procedural conditions.

The PRP agreed that the KeraSkin™ SIT RhE method adequately adheres to the essential test method components as described by OECD TG 439 and GD 220 (OECD, 2019; OECD, 2015). The PRP noted that the amount of MTT used is lower than those used within the validated reference methods (i.e., 300 µl of 0.5 mg/ml MTT (i.e., 0.15 mg MTT) for the KeraSkin™ QC procedures and 300 µl of 0.4 mg/ml MTT (i.e., 0.12 mg MTT) for the KeraSkin™ SIT method distributed as 100 µl inside the well and 200 µl outside the well of a 24 multiwell plate versus 2ml of 0.3 mg/ml MTT (i.e., 0.6 mg) for EpiSkin™ SIT and 300 µl of 1 mg/ml MTT (i.e., 0.3 mg MTT) for EpiDerm™ EPI-200-SIT). Furthermore, the volume of 1% Triton X-100 applied for the QC assessment is also lower than the usual volume applied (i.e., 50 µl versus 100 µl). However, the test developers provided evidence demonstrating that for this method, the MTT concentration and volume used did not impact the prediction obtained as compared to higher MTT concentrations. Furthermore, the test developers showed that the barrier property of the model as assessed by the ET_{50} value was comparable to those of the validated reference methods (VRMs) when tested at the same conditions (MTT concentration and Triton X-100 volume) as for the VRMs.

4. Selection of test chemicals

At least the 20 recommended reference chemicals within GD 220 should be tested with the KeraSkin™ SIT RhE method according to recommendations of paragraphs 24 to 26 of GD 220.

The PS-based validation study assessed the performance of the KeraSkin™ SIT RhE based on the 20 PS reference chemicals tested in a blind manner by three laboratories (distributed as 10 UN GHS Category 2, 3 UN GHS Category 3 and 7 UN GHS No Category). In addition, a total of 42 chemicals that did not overlap with the PS reference chemicals were tested in a blind manner (distributed as 7 UN GHS Category 2, 2 UN GHS Category 3 and 33 UN GHS No Category).

The test developers also provided information on the chemicals used for the optimisation of the test method which represented a total of 20 chemicals. These chemicals included an overlap of 6 chemicals of the 20 PS chemicals (2 UN GHS Category 2 and 4 UN GHS No Category), and 11 chemicals of the 42 additionally tested chemicals (4 UN GHS Category 2 and 7 UN GHS No Category). Based on the above information, the PRP agreed that the information provided was sufficient and adequate.

5. Study acceptance, within- and between-laboratory reproducibility

The reliability obtained with the reference chemicals for the KeraSkin™ SIT RhE method should be equal to or better than the defined minimum target values specified in paragraphs 28 and 29 of GD 220, and the analyses of reliability should be conducted according to the specifications described in paragraph 27 of GD 220. Furthermore, the tested PS reference chemicals should meet the study acceptance criteria described in paragraphs 31 and 32 of GD 220.

The PRP agreed that the information provided regarding the study acceptance criteria and the reproducibility the of the KeraSkin™ SIT RhE method was sufficient and adequate (cf. table below).

	KeraSkin™ SIT RhE method	**GD 220 target values**
Within-laboratory reproducibility	100% (20/20), 100% (20/20), 95% (19/20)	≥ 90%
Between-laboratory reproducibility	100% (20/20)	≥ 80%
% of complete run sequences / lab.	100%, 100%, 100%	≥ 85%
% of complete run sequences over the 3 labs	100%	≥ 90%

6. Predictive capacity

The predictive capacity obtained with the PS reference chemicals for the KeraSkin™ SIT RhE method should be equal to or better than the defined minimum target values specified in paragraph 30 of GD 220, and the analyses of predictive capacity should be conducted according to the specifications described in paragraph 27 of GD 220. The predictive capacity of the KeraSkin™ SIT RhE method should also be evaluated with an enlarged dataset, other than the 20 reference chemicals, and demonstrate appropriate performances.

The PRP considered the predictive capacity of the KeraSkin™ SIT RhE obtained with the 20 PS reference chemicals to be sufficient and adequate (cf. table below).

	KeraSkin™ SIT RhE method (average 3 labs)	**GD 202 target values**
Sensitivity (n=10)	**100%**	≥ 80%
Specificity (n=10)	**70%**	≥ 70%
Accuracy (n=20)	**85%**	≥ 75%

Furthermore, additional 42 chemicals tested in a blind manner. When combining their results with those obtained with the 20 reference chemicals an overall sensitivity of 100.0% (17/17), a specificity of 82.2% (37/45) and an accuracy of 87.1% (54/62) were found for the overall dataset of 62 chemicals (distributed as 17 UN GHS Category 2, 5 UN GHS Category 3 and 40 UN GHS No Category). These values were considered to be sufficient and adequate by the PRP.

7. Applicability Domain

The applicability domain of the KeraSkin™ SIT RhE method should be defined.

The PRP agreed that, based on the information provided, the applicability domain of the KeraSkin™ SIT RhE method is expected to be similar to the one of the validated reference methods.

8. Accordance with the principles of Good Laboratory Practices

All data from the PS-based validation study supporting the validity of the KeraSkin™ SIT RhE method should be obtained in accordance with the principles of Good Laboratory Practice (GLP).

The PRP agreed that the GLP-like conditions used during the PS-based validation study were sufficient and adequate. It was noted that one of the participating laboratories was GLP accredited, and the two other laboratories conducted the assay according to the principles of GLP.

9. Completeness of data and documents

Completeness of all data and documents supporting the assessment of the validity of the KeraSkin™ SIT RhE method.

The PRP agreed that the information provided was sufficient and adequate to support the assessment of the scientific validity of the KeraSkin™ SIT RhE method.

10. Validation study management and conduct

The PRP agreed that the validation study management and conduct was adequate. The PS-based validation study followed the principles of the OECD GD 34 as well as the requirements of the OECD GD 220.

11. Additional considerations

11.1. Audit of the tissues manufacturing

The PRP agreed that the additional information provided regarding the audit of the tissue manufacturing was sufficient and adequate. In particular, the tissue production is certified according to ISO 9001:2015 which requires that an external audit is conducted.

11.2. Long-distance delivery of the KeraSkin™ RhE tissues

The PRP agreed that the additional information provided on the long distance transportation of the KeraSkin™ RhE tissues was adequate. The KeraSkin™ RhE tissues were transported via air to a Chinese

laboratory where they underwent QC testing (negative control, barrier function and histology) as well as the testing of 17 PS reference chemicals showing good performances of the transported tissues.

12. Conclusions

All data should adequately support the peer review assessment that the KeraSkin™ SIT RhE method is structurally and mechanistically similar to the validated reference method, and demonstrate sufficient reliability and relevance for the proposed testing purpose i.e., that the proposed similar or modified test method is scientifically valid.

The PRP concluded that the information provided by the test method developers support the scientific validity of the KeraSkin™ SIT RhE method as a similar method, according to the OECD GD 220, to the methods included in the OECD TG 439.

IV. References

Anonym (2020). KeraSkin™ SIT Validation Report. Me-too validation study for *in vitro* skin irritation test with 3D-reconstructed human epidermis model KeraSkin™. Version from 28 July 2020.

OECD (2005). Guidance Document No. 34: Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment. Environmental Health and Safety Monograph Series on Testing and Assessment. Available at: http://www.oecd.org/env/ehs/testing/series-testing-assessment-publications-number.htm.

OECD (2015). Guidance Document No. 220: Performance Standards for the Assessment of Proposed Similar or Modified *In Vitro* Reconstructed Human Epidermis (RHE) Test Methods for Skin Irritation Testing as Described in TG 439. OECD Environmental Health and Safety Publications, Series on Testing and Assessment. Available at: http://www.oecd.org/env/ehs/testing/series-testing-assessment-publications-number.htm.

OECD (2019). OECD Guidelines for the Testing of Chemicals No. 439. *In vitro* Skin Irritation: Reconstructed Human Epidermis Test Method. Paris, France: Organisation for Economic Cooperation and Development. Originally adopted in 2010. Available at: http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788.